## REMARKS

Claims 80-99 are pending and under examination. Claim 80 has been amended. Support for the amendment can found throughout the specification and the claims as filed. In particular, support for the amendment to claim 80 can be found on page 40, line 23-30. Accordingly, this amendment does not raise an issue of new matter and entry thereof is respectfully requested.

### Priority

Applicants thank the Office for acknowledging Applicants' claim for domestic priority to the Provisional Application Serial Number 60/209,539, filed June 5, 2000 (herein "the '539 application"). However, the Office asserts that the '539 application allegedly fails to provide adequate support under 35 U.S.C. §112 for claims 80-99 for the same reasons as applied to the present application. Applicants respectfully traverse for the same reasons of record and further direct the Office to the arguments presented below with respect to the specification providing enablement for currently pending claims 80-99.

### Rejection Under 35 U.S.C. §112, First Paragraph – Enablement

The rejection of claims 80-99 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement is respectfully traversed. Applicants maintain the position of record and provide the following remarks and evidence to support that the claimed methods are enabled for their full scope.

The Office asserts that the specification fails to provide an enabling disclosure for the wide variety of progenitor cell types that may be used as starting material, such as human hematopoietic stem cells (HSCs) and human embryonic stem (ES) cells. The Office asserts that, at the time the subject application was filed, it was known in the art that difficulties were encountered in attempts to transfect human ES cells and human HSCs. The Office also asserts that methods for successfully transfecting human ES cells were not known. The Office goes on to assert that, as of June 2000, no one had successfully transfected human ES cells and that the transfection of mouse ES cells is not predictive of human ES cells. The Office also assets that the specification only provides general guidance rather than the specific guidance that is allegedly needed for human HSCs and human ES cells. The Office asserts that when methods

for the genetic modification of human ES cells are not known in the prior art, specific guidance is needed to enable the invention.

Applicants respectfully disagree with the above assertions. Applicants herein provide corroborative evidence that the specification provides sufficient teaching and guidance to one of ordinary skill in the art to make and use the claimed methods without undue experimentation. Specifically, the following remarks and evidence will demonstrate that, as of the effective filing date of the subject application, which is the filing date of the Provisional Application Serial Number 60/209,539, filed June 5, 2000, methods for transfecting human HSCs and human ES cells were well known to one of skill in the art.

Applicants respectfully submit that the specification teaches a variety of methods, known in the art, for introducing a nucleic acid into progenitor cells *in vitro* (page 54, line 4 to page 55, line 17 of the subject application and page 27, line 18 to page 28, line 19 of the '539 application). For example, the specification teaches that viral vectors can be particularly useful for introducing a nucleic acid molecule in the methods of the invention. Such vectors include, for example, retroviral vectors, lentiviral vectors, adenoviral vectors and adeno-associated vectors (AAV), herpesvirus vectors as described in text books Kaplitt and Loewy, Viral Vectors: Gene Therapy and Neuroscience Applications, Academic Press, San Diego, California (1995) and Chang, Somatic Gene Therapy, CRC Press, Boca Raton, Florida (1995) (see page 55, lines 1-10 of the subject application).

Applicants maintain the position of record regarding the disclosures of Hanazono et al. and Zwaka et al., in view of the disclosures of Eiges et al., Ferrari et al. and Uyttersprot et al. (all references are of record). Applicants also maintain that the disclosure of van Hennik et al. (also of record) shows it was well known in the art to transduce human hematopoietic cells with retroviral vectors, such as the Moloney murine leukemia virus (MoMLV). As discussed above, the specification explicitly discloses retroviral vectors as exemplary viral vectors and, as evidenced by van Hennik et al., MoMLV is a retroviral vector well known in the art as of the filing date of the subject application and the filing date of the '539 application. Therefore, one of ordinary skill in the art would understand that retroviral vectors are exemplary viral vectors that

could be used to introduce a nucleic acid molecule into progenitor cells *in vitro* based on the teachings of the subject application.

In addition to retroviral vectors, as discussed above, lentiviral vectors were also taught in the specification to be exemplary viral vectors. To corroborate that lentivral vectors were also well known in the art as suitable for introducing a nucleic acid into progenitor cells, Applicants herein provide Exhibit A, Case et al., Proc. Natl. Acad. Sci. USA, 96:2988-2993 (1999), which discloses methods for stable transduction of quiescent human hematopoietic progenitor cells by HIV-1-based lentiviral vectors (see Abstract). Specifically, Case et al. characterize their experimental results on page 2992, second column, first paragraph, as follows:

> The data presented here demonstrate that lentiviral vectors pseudotyped with the VSV envelope are able to transduce a hematopoietic progenitor population qualitatively different from that transduced by MLV retroviruses. Although both MLV and lentiviral vectors efficiently transduced CD34+ progenitors stimulated to divide during transduction, only lentiviruses could transduce more primitive, quiescent progenitors. [emphasis added]

Case et al. also disclose in the last sentence of the Abstract:

> The lentiviral vector is clearly superior to the MLV vector for transduction of quiescent, primitive human hematopoietic progenitor cells and may provide therapeutically useful levels of gene transfer in human hematopoietic stem cells. [emphasis added]

Thus, Case et al. corroborate the effectiveness of both retroviral and lentivrial vectors for transduction of human hematopoietic progenitor cells. Applicants respectfully point out that lentiviral vectors are explicitly taught in the subject application as a viral vector that can be particularly useful for introducing a nucleic acid molecule in the methods of the invention (see page 55, lines 1-10 of the subject application). Thus, Applicants submit that one of ordinary skill in the art would know how to use viral vectors, such as retroviral vectors and lentiviral vectors, as taught in the specification, to introduce a nucleic acid molecule into progenitor cells, including human hematopoietic progenitor cells.

With respect to evidence previously presented on the record to corroborate enablement, Applicants direct the Examiner to the Rule 132 Declaration of Dr. Stuart Lipton, submitted

August 29, 2008, which includes data regarding transfection of human ES cells with MEF2CA using a lentiviral vector designated lenti-MEF2CA. Exhibit B of Dr. Lipton's Declaration provides data showing the neurogenic activity of MEF2C in human ES cells. See specifically Exhibit 5, Figure 5B which shows representative images of lentivirus infected cells. The arrows in Figure 5B represent MEF2CA-induced neurons. Applicants submit that the plasmids used to generate the lenti-MEF2CA vector described in Dr. Lipton's Declaration were readily available to one of ordinary skill in the art as of the filing date of the subject application and the filing date of the '539 application.

Applicants respectfully direct the Office to Exhibit B of Dr. Lipton's Declaration, specifically Fig. 1A, the Fig. 1 legend and the first paragraph of the Methods section, under the header "Lentivirus generation and characterization," which describes the plasmids used to generate the lentiviral vector. In order to generate the lentiviral vector, three plasmids were transfected into HEK293T cells, (1) the lentiviral transfer vector: plasmid pRRLPGK/MEF2CA/IRES/GFPSIN18WPRE; (2) plasmid pCMVΔR8.74, which encodes the essential viral genes (GAG, POL, TAT, and REV) for virus production and integration and (3) plasmid pMD.G, which encodes the gene for the coat proteins of Vesicular stomatitis Virus-G (VSV-G). The lenti-MEF2CA vector was produced in these cells and used in the experiments described. Applicants submit that plasmids pCMVΔR8.74 and pMD.G are known packaging constructs available as of the filing date of the subject application and the filing date of the '539 application.

Applicants herein provide Exhibit B, Dull et al., J. Virol., 72(11):8463-8471 (1998), which discloses a third-generation lentivirus vector with a conditional packaging system. Specifically, Dull et al. disclose that plasmids pCMVΔR8.74 and pMD.G are known packaging constructs used to generate lentiviral vectors (see page 8464, first column, paragraphs under the heading "Packaging constructs"). Applicants also herein provide Exhibit C, Naldini et al., Science, 272:263-267 (1996), which discloses the gene delivery and stable transduction of nondividing cells by a lentiviral vector based on the human immunodeficiency virus (HIV). Specifically, Applicants direct the Office to Figure 1, page 263, first column, last paragraph to third column, first paragraph, and page 266, third column, citation 14, which describe a three plasmid expression system including the plasmids pCMVΔR9 and pMD.G. Thus, Applicants

submit that Dr. Lipton's Declaration provides corroborative evidence that nucleic acids were able to be introduced into human ES cells, using methods readily available to one of ordinary skill in the art as of the filing date of the subject application and the filing date of the '539 application. Thus, the evidence provided in Dr. Lipton's declaration is consistent with the teachings in the specification that lentiviral vectors are effective viral vectors for introducing a nucleic acid molecule in to progenitor cells and utilized methods for lentivral transduction well known prior to the filing date of the subject application and the filing date of the '539 application. As stated in the Office Action on page 13, "sufficiency under 35 U.S.C. 112 must be judged as of the filing date." Accordingly, Applicants submit that one of ordinary skill in the art at the time of filing the subject application and the '539 application would only need to use known techniques and routine experimentation, as taught in the specification, to introduce a nucleic acid molecule into progenitor cells, including human embryonic stem cells.

Applicants submit that the specification fully satisfies the requirement for enablement under 35 U.S.C. §112, first paragraph for the claimed methods. "The law does not require a specification to be a blueprint in order to satisfy the enablement requirement," *Staehelin v. Secher*, 24 U.S.P.Q. 2d 11513, 1516 (Bd. Pat. App. & Int. 1992). A specification need not describe—and best omits—that which is well known in the art. *In re Buchner*, 929 F.2d 660, 661, 18 U.S.P.Q.2d 1331, 1332 (Fed. Cir. 1991). Furthermore, the first paragraph of 35 U.S.C. §112 requires nothing more than objective enablement. *In re Marzocchi*, 439 F.2d 220, 169 USPQ 367 (CCPA 1971). "How such a teaching is set forth, either by the use of illustrative examples or by broad terminology, is of no importance when determining enablement." *Id* at 223. It is also well-settled in the law that "a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *Ex parte Jackson*, 217 U.S.P.Q. 804, 807 (Bd. App. 1982). As discussed above and corroborated by the evidence of record and the evidence submitted herewith, only well known methods and nothing more than routine experimentation would have been required for one skilled in the art to make and use Applicants' claimed methods.

In view of the foregoing arguments and for the reasons of record, Applicants maintain that, in light of the teaching in the specification, what was well known in the art and the

corroborative evidence of record, the specification provides sufficient teaching and guidance to enable the claimed methods. Accordingly, Applicants respectfully request withdrawal of this rejection.

**Rejections Under 35 USC § 102**

The rejection of claims 80-99 under 35 USC § 102(a), as allegedly anticipated by Okamoto et al., *Proc. Natl. Acad. Sci. USA* 97:7561-7566 (2000), is respectfully traversed.

Applicants respectfully maintain that the claimed methods are novel over Okamoto et al. Applicants further point out that the priority application, serial No. 60/209,539, was filed June 5, 2000, which provides an enabling disclosure for the claimed methods as described above. The Office Action states on page 17 that the rejection stands or falls with the enablement rejection. Applicants respectfully maintain that the priority date of the subject application is prior to the publication date of Okamoto et al. In particular and as discussed above, Applicants maintain that the priority '539 application provides sufficient description and guidance to enable the claimed methods. Therefore, Applicants respectfully submit that Okamoto et al. is not proper prior art. Accordingly, Applicants respectfully request that this rejection be withdrawn.

The rejection of claims 80-99 under 35 U.S.C. § 102(b) as allegedly anticipated by Krainc et al., *J. Biol. Chem.* 273:26218-26224 (1998), is respectfully traversed. Applicants respectfully maintain that the claimed methods are novel over Krainc et al. for the reasons of record and in view of the following.

The Office asserts that Krainc et al. disclose that the plasmid pG/DN, containing the N-terminal DNA binding domain of MEF2C, was stably transfected into P19 cells. The Office also asserts that Krainc et al. note that these cells differentiate into a neuronal phenotype after treatment with retinoic acid, and then express MEF2C. The Office goes on to assert that the N-terminal fragment of MEF2C used in the experiments of Krainc et al. contain the entire DNA-binding domain of the MEF2C protein and therefore is both constitutively active for DNA binding and an active fragment of a MEF2 polypeptide, as set forth in the claims. The Office also asserts that there is no evidence demonstrating that the MEF2C-transfected P19 progenitor cells of Krainc et al. are functionally different from those recited in the claims.

Applicants respectfully disagree with the above assertions. Applicants maintain that Krainc et al. do not teach, either expressly or inherently, each element of the claimed methods. Applicants direct the Office to the teachings of the subject application, which defines a constitutively active MEF2 polypeptide on page 40, line 23-30 as follows:

> As used herein in reference to a MEF2 polypeptide, the term "constitutively active" means a MEF2 polypeptide that has transactivation activity which is less dependent upon phosphorylation than the corresponding wild type MEF2 polypeptide. A constitutively active MEF2 polypeptide can have transactivation activity that is independent of phosphorylation. [emphasis added]

Thus, one of ordinary skill in the art, in light of the teachings of the specification, would understand that a constitutively active MEF2 polypeptide is an MEF2 polypeptide, which has transcriptional activation activity that is constitutively active and is independent of phosphorylation. Furthermore, Applicants maintain that one skilled in the art would not consider a "dominant negative" as described by Krainc et al. to be "constitutively active." Nevertheless, in an effort for further prosecution, Applicants have amended claim 80 to explicitly recite "wherein said constitutively active MEF2 polypeptide or active fragment thereof have transactivation activity that is independent of phosphorylation."

Applicants respectfully submit that the nucleic acids encoding the N-terminal fragment of MEF2C protein used by Krainc et al. is not a nucleic acid molecule encoding a constitutively active MEF2 polypeptide or an active fragment thereof, wherein the constitutively active MEF2 polypeptide or active fragment thereof have transactivation activity that is independent of phosphorylation, as claimed. At best, Krainc et al. disclose on page 26222, column 2, paragraph 2, that P19 cells were stably transfected with the plasmid pG/DN encoding a dominant-negative MEF2C, which contains the cDNA sequence of the N-terminal DNA binding domain of MEF2C. Krainc et al. describe the pG/DN plasmid on page 26219, first column, first paragraph, as carrying a mouse dominant-negative MEF2C (amino acids 1-108) driven by the phosphoglycerate kinase promoter, which was a gift from Dr. E. N. Olson (34). Reference (34) is herein provided as Exhibit D, Molkentin et al., *Mol. Cell. Biol.* 16(6):2667-2636 (1996). Exhibit D describes the mutational analysis of the DNA binding, dimerization and transcriptional activation domains of MEF2C. Applicants direct the Office to Figures 1A and Figure 2, which show that polypeptides encoding N-terminal DNA binding domains and MEF2C dimerization

domains have significantly reduced transcriptional activation activity. The authors of Exhibit D characterize these results on page 2634, second column, first paragraph under the heading "Interdependence of MEF2 domain and the transcription activation domain" as follows:

> Our results demonstrate that the C terminus of MEF2C acts as a transcription activation domain. The first 105 amino acids of MEF2C, which encompass the MADS and MEF2 domains, can dimerize and bind DNA, but this region is unable to activate transcription through the MEF2 site in the absence of a C-terminal transcription activation domain.

Thus, Applicants submit that the nucleic acids encoding the N-terminal fragment of MEF2C protein used by Krainc et al. was not a constitutively active MEF2 polypeptide or active fragment thereof as recited in the pending claims, but was the N-terminal region of the MEF2C polypeptide that is unable to activate transcription. Applicants respectfully submit that the disclosure of Krainc et al. cannot anticipate the claimed methods because the reference does not teach, either expressly or inherently, each element of the claimed methods. Accordingly, Applicants respectfully request that this rejection be withdrawn.

## CONCLUSION

In light of the amendments and remarks herein, Applicants submit that the claims are now in condition for allowance and respectfully request a notice to this effect. The Examiner is invited to call the undersigned agent if there are any questions.

To the extent necessary, a petition for an extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due in connection with the filing of this paper, including extension of time fees, to Deposit Account 502624 and please credit any excess fees to such deposit account.

Respectfully submitted,

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EXHIBIT A

*Proc. Natl. Acad. Sci. USA*
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Immunology

# Stable transduction of quiescent CD34$^+$CD38$^-$ human hematopoietic cells by HIV-1-based lentiviral vectors

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*Edited by Inder M. Verma, The Salk Institute for Biological Studies, San Diego, CA, and approved January 18, 1999 (received for review November 9, 1998)*

**ABSTRACT** **We compared the efficiency of transduction by an HIV-1-based lentiviral vector to that by a Moloney murine leukemia virus (MLV) retroviral vector, using stringent *in vitro* assays of primitive, quiescent human hematopoietic progenitor cells. Each construct contained the enhanced green fluorescent protein (GFP) as a reporter gene. The lentiviral vector, but not the MLV vector, expressed GFP in nondivided CD34$^+$ cells (45.5% GFP$^+$) and in CD34$^+$CD38$^-$ cells in $G_0$ (12.4% GFP$^+$), 48 hr after transduction. However, GFP could also be detected short-term in CD34$^+$ cells transduced with a lentiviral vector that contained a mutated integrase gene. The level of stable transduction from integrated vector was determined after extended long-term bone marrow culture. Both MLV vectors and lentiviral vectors efficiently transduced cytokine-stimulated CD34$^+$ cells. The MLV vector did not transduce more primitive, quiescent CD34$^+$CD38$^-$ cells ($n = 8$). In contrast, stable transduction of CD34$^+$CD38$^-$ cells by the lentiviral vector was seen for over 15 weeks of extended long-term culture (9.2 ± 5.2%, $n = 7$). GFP expression in clones from single CD34$^+$CD38$^-$ cells confirmed efficient, stable lentiviral transduction in 29% of early and late-proliferating cells. In the absence of growth factors during transduction, only the lentiviral vector was able to transduce CD34$^+$ and CD34$^+$CD38$^-$ cells (13.5 ± 2.5%, $n = 11$ and 12.2 ± 9.7%, $n = 4$, respectively). The lentiviral vector is clearly superior to the MLV vector for transduction of quiescent, primitive human hematopoietic progenitor cells and may provide therapeutically useful levels of gene transfer into human hematopoietic stem cells.**

Gene therapy using human hematopoietic stem cells (HSC) has great theoretical appeal as an approach to many genetic and acquired diseases affecting the hematopoietic and immune systems. However, progress in the field has been blocked by the fact that levels of gene transfer into human long-term repopulating cells are too low for any likely therapeutic benefit (1–5). The reason for the disappointingly low levels of transduction is believed to lie in certain incompatible features of the vectors used and the HSC that they target. Vectors for hematopoietic gene therapy have until now been based on the Moloney murine leukemia virus (MLV) and are thus unable to infect and integrate into nondividing cells (6). Most HSC are in a quiescent state (7), are relatively slow to respond to stimulation (8–12), and, when induced to divide, tend to lose long-term repopulating capacity (12–17). In addition, the relative paucity of viral receptors on the surface of HSC may limit binding of virus and further prevent efficient gene transfer (18, 19).

Recent incremental improvements in MLV retroviral-mediated gene transfer into HSC have been achieved by using gibbon ape leukemia virus (GALV) pseudotypes, "mobilized bone marrow," recombinant fibronectin support, new cytokines (Flt-3 ligand, thrombopoietin), and manipulation of cell cycle kinetics (14, 20–23). Combinations of these techniques have resulted in modest, yet significant, increases in gene marking in primate stem cell transplant models. However, higher levels of gene transduction of HSC are likely to be needed for applications to many genetic diseases and AIDS.

Recent reports show that vectors derived from the HIV-1 lentivirus can transduce a variety of nondividing human cells, including neurons, macrophages, hepatocytes, and cardiac myocytes (24–32). The nuclear localization signals present in HIV allow entry of virus through the intact nuclear membrane of nondividing cells (33). Pseudotyping of lentivirus vector with the vesicular stomatitis virus (VSV) envelope G glycoprotein allows virus particles to bind nonspecifically to membrane phospholipid of target cells rather than relying on specific receptor binding (34). Thus, lentiviral vectors pseudotyped with VSV offer a potential solution to the dual problems of quiescence and low viral receptor expression inherent in transduction of HSC with MLV.

We show that lentiviral vectors, but not MLV vectors, can transduce nondivided hematopoietic progenitors and CD34$^+$CD38$^-$ cells in $G_0$ cell cycle status. Using stringent long-term culture (LTC) and single-cell assays, we show that lentiviral vectors are able to provide efficient, stable transduction in primitive, quiescent human progenitors normally resistant to transduction with MLV.

## MATERIALS AND METHODS

**Production and Characterization of Vectors.** The MLV retroviral vector, MLV-Neo-CMV-GFP (35), and the lentiviral vector, pHR′-CMV-GFP (24, 27), were constructed as described and contained the enhanced green fluorescent protein (GFP; CLONTECH) reporter gene with the internal human cytomegalovirus (CMV) immediate-early promoter. The plasmid pHIT60 (36) was used to express the MLV gag-pol proteins. The plasmid pCMVΔR8.91 (28) was used to express HIV-1 gag, pol, tat, and rev proteins to package lentiviral vectors without the accessory genes *vif, vpu, vpr,* and *nef*. An integration-defective lentiviral vector was generated as described (24, 26). The plasmid pMD.G (24) was used to

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: MLV, Moloney murine leukemia virus; GFP, enhanced green fluorescent protein; LTC, long-term culture; ELTC, extended LTC; ELTC-IC, ELTC-initiating cell; VSV, vesicular stomatitis virus; GALV, gibbon ape leukemia virus; i.u., infectious unit; moi, multiplicity of infection; CFU, colony-forming units; HSC, hematopoietic stem cell; FACS, fluorescence-activated cell sorting; IL, interleukin; SF, Steel factor; DAPI, 4′,6-diamidino-2-phenylindole.

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express the VSV envelope G glycoprotein from the CMV immediate-early promoter.

The lentiviral vector, the integrase-defective lentiviral vector, and the MLV vector were all pseudotyped with the VSV envelope (lenti/VSV, lenti(int$^-$)/VSV, and MLV/VSV, respectively). VSV-pseudotyped vectors were produced by transient three-plasmid transfection as previously described (24) with 2 $\mu$g of the pMD.G envelope plasmid and 10 $\mu$g of the various packaging and vector plasmids. Sodium butyrate (Sigma) induction was performed as described (36). Preparations of VSV-pseudotyped vectors were concentrated by ultracentrifugation (37). Another MLV vector, MND-GFP-SV40-Neo, was produced in a GALV pseudotype (MLV/GALV) from the stable packaging cell line PG13 (38).

Titers of all vector preparations were determined by transducing 293 cells (American Type Culture Collection) with serial dilutions of vector supernatants, followed by fluorescence-activated cell sorting (FACS) analysis 2 days later. Initial titers were $0.5 \times 10^6$ to $10 \times 10^6$ infectious units (i.u.)/ml for the lenti/VSV, lenti(int$^-$)/VSV, MLV/VSV, and MLV/GALV vectors. After ultracentrifugation, the titers of the VSV pseudotyped lentiviral and MLV vectors were $1–15 \times 10^8$ i.u./ml.

All lentiviral vector preparations were tested for the presence of replication-competent retrovirus (RCR) by infection of phytohemagglutinin (PHA)-stimulated human peripheral blood mononuclear cells, followed by culture for 2 weeks and then assay of culture medium for p24 gag by ELISA (Coulter). No vector preparations contained detectable RCR.

**Cell Sources and Isolation.** Mononuclear cells from fresh bone marrow and umbilical cord blood were obtained as previously described under protocols approved by the Committee on Clinical Investigations (39). FACS was performed on a FACSVantage [Becton Dickinson Immunocytometry Systems (BDIS)] using LYSYS II software (BDIS). CD34$^+$CD38$^-$ cells were defined as previously described (39). CD34$^+$ cells were defined as either cells with high CD34 expression alone, or in some experiments cells with high CD34 and CD38 expression (CD34$^+$CD38$^+$), as previously described (39).

**Multiparameter Cell Cycle Analysis.** CD34$^+$ cells ($3.4 \times 10^6$) were transduced in 6 ml of X-vivo 15 (BioWhittaker) containing 0.5 ng/ml interleukin (IL)-3 and 25 units/ml Flt-3 ligand in flasks coated with the recombinant fibronectin fragment CH-296 (Takara Shuzo, Otsu, Shiga, Japan). Viral supernatants were added daily to the cells on 3 consecutive days. lenti/VSV transduction was performed with the supernatant concentration at $1 \times 10^7$ i.u./ml [equivalent multiplicity of infection (moi) = 18] each day. MLV/GALV transduction was performed with the supernatant concentration at $5 \times 10^5$ i.u./ml (moi = 0.9) each day. Mock-infected (nontransduced) controls were handled exactly the same, but with no vector supernatants added to the CD34$^+$ cells.

Cell cycle activity and transgene expression were analyzed 24 hr after the third addition of virus by employing a modification of a previously described flow cytometric procedure (40). The revised protocol allows the use of an additional fluorochrome and is hence referred to as five-color SID (surface, intracellular, DNA) staining. Specifically, cells were labeled with anti-CD34-biotin (Coulter), streptavidin-Red613 (GIBCO/BRL), and anti-CD38-APC (BDIS; APC is allophycocyanin). Cells were then fixed in 0.5% formaldehyde (Polysciences) and permeabilized with 0.1% Triton X-100 (Amersham), Ki-67-PE (Dako; PE is phycoerythrin) was added, and finally 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) was added (2 $\mu$M) to stain for DNA content. Analysis was performed on a Becton Dickinson FACSVantage flow cytometer. DNA level was measured by excitation of DAPI from the 350-nm line, PE and Red613 were excited by the 488-nm line, and APC by the 633-nm line. Transduction of primitive cells was detected by the presence of GFP (488-nm laser). The nuclear antigen Ki-67 was used as a marker of cell cycle entry (41, 42) and was used with DAPI to delineate $G_0$ and $G_1$ populations and cycling (S, $G_2$, M) stages.

**Analysis of Transgene Expression in Nondivided Cells.** CD34$^+$ cells ($1–2 \times 10^6$) were incubated in 2 ml of diluent with the red fluorescent membrane marker PKH26 (final concentration $2 \times 10^{-6}$ M; Sigma) for 1.5 min at room temperature. Then 10% fetal calf serum (FCS; Summit Biotechnology, Fort Collins, CO) was added to block further adsorption of dye and the cells were washed four times. A narrow band of viable PKH26-bright (nondivided) cells was isolated by FACS after overnight culture on CH-296 and immediately transduced once with lenti/VSV, lenti(int$^-$)/VSV, or MLV/GALV on CH-296 in X-vivo 15 with 2.5 ng/ml IL-3, 8.25 units/ml IL-6, and 12.5 ng/ml Steel factor (SF). After 24 hr, cells were washed twice and incubated in the absence of vector for a further 24 hr on fresh CH-296 in the same culture conditions. A total of 48 hr after transduction, cells were washed and incubated with anti-CD34-APC (BDIS). Cells were analyzed by FACS for simultaneous PKH26, GFP, and CD34 expression. PKH26 fluorescence in nondivided cells remained identical between the first isolation and the time of final analysis. The width of the PKH26 band set for each generation was identical.

**Transduction of Hematopoietic Cells Before LTC.** Hematopoietic cell transductions were performed in plates coated with CH-296. CD34$^+$ cells ($1–10 \times 10^4$ per plate) were transduced in 2–4 ml of diluent in 35-mm plates (Costar). CD34$^+$CD38$^-$ cells ($3–30 \times 10^2$ per well) were transduced in 200 $\mu$l of X-vivo 15 in 96-well plates (Costar). Transductions were performed with lenti/VSV, lenti(int$^-$)/VSV, and MLV/VSV, using equivalent supernatant concentrations of $3–15 \times 10^6$ i.u./ml (moi = 1,000–3,000). Transductions with MLV/GALV were performed with supernatant concentrations of $5–18 \times 10^5$ i.u./ml (moi = 100–300).

Transductions carried out in the presence of growth factors (5 ng/ml IL-3, 16.5 units/ml IL-6, and 25 ng/ml SF) were performed with one addition of viral supernatant per day for either 1 day or for 3 consecutive days. As there was no significant difference in the results with the two protocols, the data were grouped together. Transductions carried out in the absence of growth factors were performed with one addition of viral supernatant for 12 hr immediately after isolation. After transduction, cells were placed in LTC for serial analysis.

**Analysis of LTCs.** CD34$^+$CD38$^-$ cells were cultured long-term (approximately 100 days) on irradiated allogeneic human bone marrow stroma in long-term bone marrow culture (LTBMC) medium (39). Every 2–3 weeks, nonadherent cells were analyzed by FACS for transgene expression or were plated in methylcellulose medium to measure colony-forming units (CFU) (39). CFU generated from the LTC were individually analyzed for GFP expression by using a fluorescent microscope, and they were isolated from the methylcellulose and analyzed by polymerase chain reaction (PCR) for detection of vector DNA, as described below.

**Clonal Analysis of Transduced Single CD34$^+$CD38$^-$ Cells.** CD34$^+$ cells were transduced with lenti/VSV, MLV/VSV, or MLV/GALV on CH-296 in the presence of IL-3, IL-6, and SF for 1 day. After transduction, cells were washed three times and incubated with anti-CD34-PE (BDIS) and anti-CD38-APC. Single CD34$^+$CD38$^-$ cord blood cells were isolated and plated in each well of a 96-well plate by FACS using the automated cell deposition unit (ACDU) device on the FACSVantage and grown on irradiated human stroma in LTBMC medium. Wells were observed every 7 days for the first appearance of clonal proliferation as described (39). GFP expression of clones was assessed by fluorescence microscopy and also by FACS analysis.

**Detection of Vector DNA.** The presence of vector sequences in extracted DNA from bulk LTC was determined by using

semiquantitative DNA PCR and Southern blot analysis for GFP. A clone of 293 cells with a single integrated copy of the pHR′-CMV-GFP vector was used to construct a standard curve for GFP normalized against human β-actin (D.B.K., unpublished work). Vector DNA in individual CFU was measured by PCR of whole cell lysate followed by Southern blot detection of GFP transgene.

**Statistical Analyses.** Statistical analyses of vector-transduced $CD34^+$ and $CD34^+CD38^-$ cells used a two-sample paired Student's *t* test assuming unequal variances as the number of experiments varied. Vector expression in clones from single $CD34^+CD38^-$ cells was analyzed by using a paired two-sample Student's *t* test.

## RESULTS

**Transduction of $CD34^+CD38^-$ Cells in $G_0$ by Lentiviral Vectors.** To determine if an HIV-1-based lentiviral vector pseudotyped with VSV was capable of transducing $CD34^+CD38^-$ cells in $G_0$ phase of the cell cycle, five-color SID staining was performed. Cells were transduced in conditions designed to minimize cell cycling. 12.4% of $CD34^+CD38^-$ cells in $G_0$, 12.9% in $G_1$, and 21% in $S/G_2/M$ phase were $GFP^+$ 24 hr after exposure to lenti/VSV, showing similar levels of transduction in cells at different stages of the cell cycle (Fig. 1). In contrast, exposure to MLV/GALV resulted in barely detectable levels of GFP expression in $CD34^+CD38^-$ cells in $G_0$ and $G_1$ phase. However, once the cells entered $S/G_2/M$ phase, GFP expression from MLV/GALV was detectable in 7.2% of $CD34^+CD38^-$ cells. These findings indicate that transgene expression in noncycling $CD34^+CD38^-$ cells is possible with lentiviral vectors but not with MLV vectors.

**Transduction of Nondivided $CD34^+$ Cells by Lentiviral Vectors.** To determine if lentiviral vectors were capable of transducing hematopoietic progenitors prior to cell division, $CD34^+$ cells were stained with PKH26. MLV/GALV was unable to transduce $CD34^+$ cells prior to cell division, but it was able to transduce cells after the first, second, and third divisions at progressively increasing levels (Fig. 2*A*). In contrast, both nondivided and divided populations of $CD34^+$ cells transduced with lenti/VSV expressed GFP at high levels, with nondivided, first, second, and third divisions at 45.5%, 44.8%, 56%, and 77.2% $GFP^+$, respectively (Fig. 2*B*).

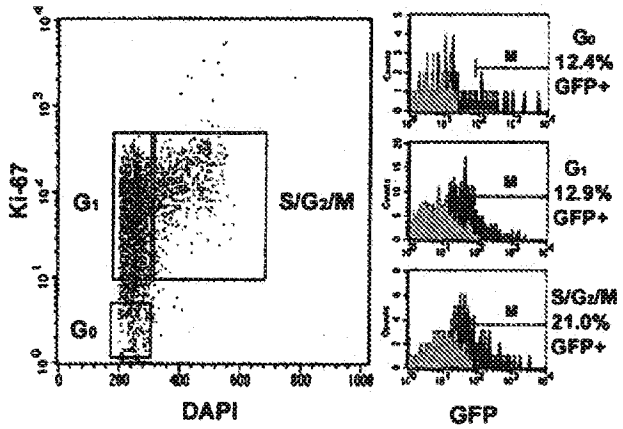


FIG. 1. Lentiviral vector expression in $CD34^+CD38^-$ cells defined according to cell cycle status. Cell cycle analysis of cells derived from the $CD34^+CD38^-$ gate (not shown) and simultaneous GFP expression of $G_0$, $G_1$, and $S/G_2/M$ populations are shown. Black histogram = transduced cells, gray histogram = nontransduced cells (negative control). Percent $GFP^+$ was calculated by subtracting the negative control cells falling within the marker region. A second experiment yielded similar results.

To determine whether GFP expression indicated integration of lentivirus vector, $CD34^+$ cells were also transduced with lenti($int^-$)/VSV. As shown in Fig. 2*C*, lenti($int^-$)/VSV resulted in 0.7% GFP expression in nondivided $CD34^+$ cells. In a second experiment (not shown), 38.5% of nondivided cells were $GFP^+$. The average level of GFP expression from the integrase-defective vector was significantly lower than that expressed from the wild-type vector. These results imply that early scoring of transgene expression in lenti/VSV-transduced cells can detect pseudotransduction and/or transient expression from nonintegrated vector DNA, and in the absence of other data may not be fully predictive of stable transduction.

To determine the stability of GFP expression from lenti/VSV- and lenti($int^-$)/VSV-transduced $CD34^+$ cells, nondivided $CD34^+$ cells were isolated and analyzed after a further 7 days of *in vitro* culture. Although the nondivided $CD34^+$ cells transduced by lenti/VSV were originally 45.5% $GFP^+$, expression fell to 3.7% at day 7. GFP was not detected in lenti($int^-$)/VSV-transduced $CD34^+$ cells after 7 days of culture. Comparable data were obtained in a second experiment.

**Transduction of $CD34^+$ Cells by Lentiviral Vectors.** Since GFP protein was detectable in $CD34^+$ cells exposed to nonintegrating lentiviral vector, long-term assays were required to evaluate stable integration of lentiviral vectors in human hematopoietic cells. As shown in Fig. 3*A*, all three vectors (lenti/VSV, MLV/GALV, and MLV/VSV) were able to transduce $CD34^+$ cells, a largely cycling, committed progenitor population, in the presence of growth factors. At 6 days after transduction (in most cases with a single exposure to virus), GFP expression with lenti/VSV was 24.4 ± 3.7% ($n$ = 11), with MLV/GALV it was 18.5 ± 7.4% ($n$ = 6), and with MLV/VSV it was 4.3 ± 1.7% ($n$ = 5) ($P$ = 0.25 for MLV/GALV and $P$ = 0.0003 for MLV/VSV, compared with lenti/VSV). The reason for the low efficiency of transduction of $CD34^+$ cells with MLV/VSV, despite high titers on 293 cells, is unclear but may be inefficient MLV vector processing after endocytosis of the VSV-pseudotyped particle.

Only lenti/VSV produced relatively stable GFP expression (16.4 ± 2.8%) over 5 weeks of culture. GFP expression from cells transduced by either MLV/VSV or MLV/GALV fell to approximately 1% by 5 weeks ($P$ = 0.002 for MLV/GALV and $P$ = 0.003 for MLV/VSV, compared with lenti/VSV). $CD34^+$ cells exposed to lenti($int^-$)/VSV showed no GFP expression in LTC.

Growth factors (e.g., IL-3, IL-6, and SF) are routinely used to induce $CD34^+$ cell cycling to achieve MLV transduction but may also induce differentiation and loss of long-term engrafting capacity (43, 44). Therefore, we determined whether lentiviral vectors could transduce $CD34^+$ cells in the absence of growth factors with brief (12-hr) exposure to virus. As shown in Fig. 3*B*, the lentiviral vector, but not the MLV vectors, was able to transduce $CD34^+$ cells in these conditions. At 6 days after transduction, GFP expression with lenti/VSV was 9.9 ± 1.6% ($n$ = 11), with MLV/GALV it was 0.6 ± 0.2% ($n$ = 8), and with MLV/VSV it was 0.2 ± 0.1% ($n$ = 7) ($P$ = 0.0002 for MLV/GALV and $P$ = 0.0001 for MLV/VSV, compared with lenti/VSV). Once again, lenti/VSV produced stable GFP expression of 13.5 ± 2.5% for 5 weeks of culture, whereas the two MLV vectors produced no long-term expression ($P$ = 0.0004 for either MLV/GALV or MLV/VSV, compared with lenti/VSV).

**Transduction of $CD34^+CD38^-$ Cells by Lentiviral Vectors.** We next studied lentiviral transduction of $CD34^+CD38^-$ cells, a more primitive progenitor population almost entirely in $G_0$ which contains HSC and is relatively resistant to MLV vector transduction (39, 40). As shown in Fig. 3*C*, the lentiviral vector was able to efficiently transduce $CD34^+CD38^-$ cells at levels similar to the level for $CD34^+$ cells, while the MLV vectors produced low to undetectable levels of GFP early in culture. At 30 days after transduction, GFP expression with lenti/VSV was 15.6 ± 2.7% ($n$ = 7), with MLV/GALV it was 0.1 ± 0% ($n$ = 4), and with

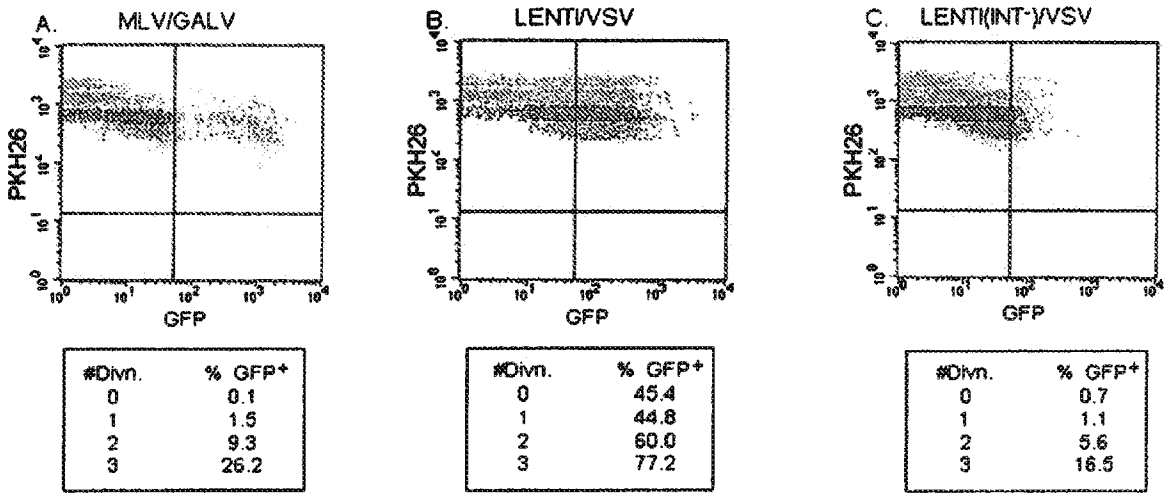


FIG. 2. Vector expression in nondivided and divided CD34⁺ cells. PKH26 fluorescence is brightest in nondivided cells and decreases with each cell division. Cells that retain their original level of PKH26 fluorescence have not divided. Shown is GFP expression of four generations of CD34⁺-gated cells. Each generation is represented by a different color in the dot plots, green indicating nondivided cells. (*A*) MLV/GALV. (*B*) lenti/VSV. (*C*) lenti(int⁻)/VSV. (*Lower*) Percent GFP⁺ cells is shown for each generation.

MLV/VSV it was 2.9 ± 2.8% ($n$ = 4) ($P$ = 0.002 for MLV/GALV and $P$ = 0.01 for MLV/VSV, compared with lenti/VSV). Only lenti/VSV produced stable GFP expression (9.2 ± 5.2%) in extended LTC (ELTC) ($P$ = 0.03 for either MLV/GALV or MLV/VSV, compared with lenti/VSV). Semiquantitative PCR analysis of DNA from nonadherent cells from LTC demonstrated that the lentiviral vector had efficiently transduced primitive progenitors (0.2–2.4 vector copies per cell at weeks 6–8) and confirmed the absence of vector DNA in LTC from CD34⁺CD38⁻ cells exposed to MLV.

ELTC-initiating cells (ELTC-IC) are a subpopulation of CD34⁺CD38⁻ cells that are quiescent and pluripotent and proliferate late in culture, generating CFU beyond 60 days and forming cobblestone areas after 30 days (11, 39, 45). As shown in Table 1, the ability of lenti/VSV to transduce ELTC-IC was confirmed by the presence of GFP⁺ CFU at 6, 8, and 10 weeks

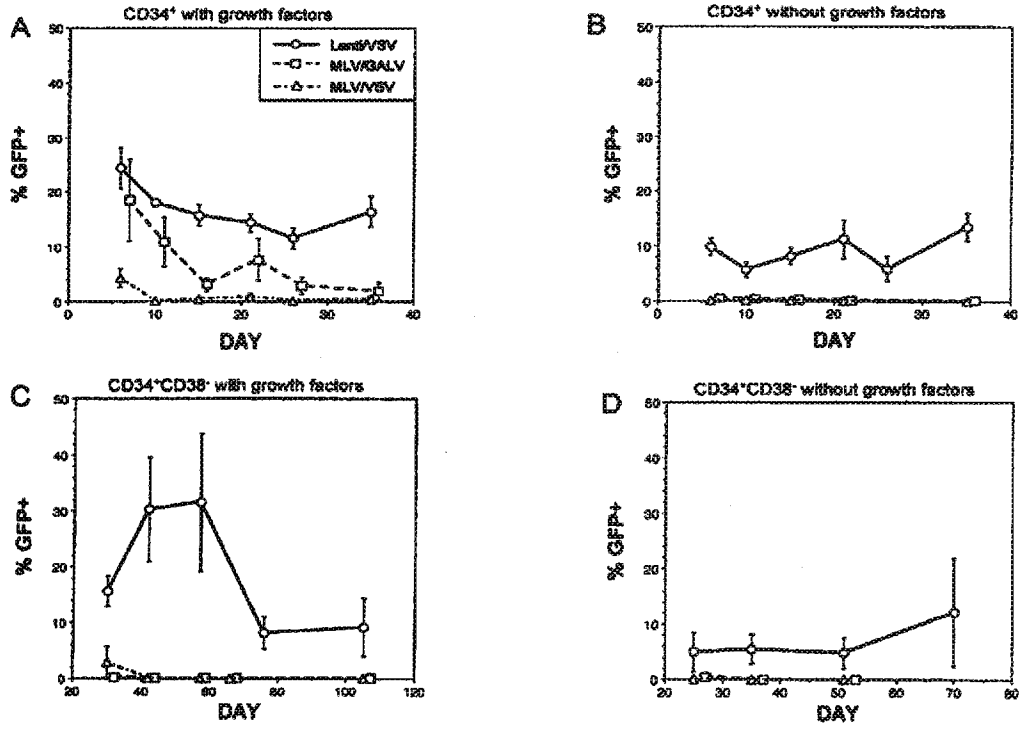


FIG. 3. Vector expression in transduced CD34⁺ and CD34⁺CD38⁻ cells. FACS analysis was performed on LTC of transduced cells at the time points indicated. Mean ± SEM for all experiments is shown. For all panels, the legend of vectors is as follows: lenti/VSV = solid line with ○; MLV/GALV = dashed line with □, and MLV/VSV = broken-dashed line with △. (*A*) CD34⁺ cells transduced with growth factors by lenti/VSV ($n$ = 11), MLV/GALV ($n$ = 6), and MLV/VSV ($n$ = 5). (*B*) CD34⁺ cells transduced in the absence of growth factors by lenti/VSV ($n$ = 11), MLV/GALV ($n$ = 8), and MLV/VSV ($n$ = 7). (*C*) CD34⁺CD38⁻ cells transduced with growth factors by lenti/VSV ($n$ = 7), MLV/GALV ($n$ = 4), and MLV/VSV ($n$ = 4). (*D*) CD34⁺CD38⁻ cells transduced in the absence of growth factors by lenti/VSV ($n$ = 4), MLV/GALV ($n$ = 4), and MLV/VSV ($n$ = 2).

Table 1. GFP transgene expression and vector DNA detection from lenti/VSV-transduced CFU

| Exp. | Week | % GFP$^+$ | % DNA$^+$ |
|---|---|---|---|
| 1 | 8 | 35 (6/17) | 47 (8/17) |
| 2 | 6 | 50 (17/34) | 91 (31/34) |
| | 10 | 96 (46/48) | 94 (45/48) |

CFU arising from LTC at weeks 6–10 were analyzed for GFP expression and vector DNA. In parentheses is the number of GFP$^+$ or DNA$^+$ CFU/the total number of CFU.

of ELTC. PCR of individual CFU arising after 6 weeks of LTC confirmed the presence of the transgene in lentiviral vector-transduced CD34$^+$CD38$^-$ cells. Thus, lentiviral vectors and not MLV vectors are able to transduce ELTC-IC (11).

To provide the most stringent test of transduction of quiescent cells, CD34$^+$CD38$^-$ cells were briefly exposed to virus in the absence of growth factors. As shown in Fig. 3*D*, only lenti/VSV ($n$ = 4) was able to transduce CD34$^+$CD38$^-$ cells without growth factor stimulation (5 ± 3.5% at 25 days) with persistent GFP expression late in culture (12.2 ± 9.7% at 10 weeks). As expected, both MLV/GALV ($n$ = 4) and MLV/VSV ($n$ = 2) were unable to transduce CD34$^+$CD38$^-$ cells without growth factors. Again, semiquantitative PCR analysis of DNA from nonadherent cells from LTC demonstrated the high transduction efficiency of CD34$^+$CD38$^-$ cells by lentiviral vectors (0.4–1.3 copies per cell at weeks 7–10) and confirmed the absence of vector DNA in CD34$^+$CD38$^-$ cells transduced by MLV.

**Clonal Analysis of CD34$^+$CD38$^-$ Cells Transduced by Lentiviral Vectors.** To analyze the stable transduction of clonogenic CD34$^+$CD38$^-$ cells on a single-cell level, CD34$^+$CD38$^-$ cells were isolated after one exposure to virus and plated in individual wells. New colonies that appeared each week were scored for GFP expression by fluorescent microscopy and FACS analysis. Late-appearing clones (those appearing after 4 weeks in culture) are the equivalent of ELTC-IC (9, 11). As shown in Table 2, MLV/GALV was able to transduce 2% (2/124) of the total cells that formed colonies, all of which appeared within the first 2 weeks (2/80, or 3%) and thus were generated from early proliferating cells. MLV/VSV was unable to transduce any of the CD34$^+$CD38$^-$ cells. In contrast, lenti/VSV was able to transduce 29% (83/285) of the total clones that formed colonies, with comparable transduction efficiencies for early and late-appearing clones. Thus, lenti/VSV provided efficient stable transduction of both proliferating and quiescent primitive CD34$^+$CD38$^-$ cells.

## DISCUSSION

A major technical problem revealed in all clinical gene therapy trials using MLV vectors has been the ability of MLV to efficiently transduce mature committed human hematopoietic progenitor cells but not pluripotent long-term repopulating HSC (46). Transduction of HSC is necessary to achieve enduring production of genetically corrected hematopoietic and lymphoid cells in a clinical setting. The data presented here demonstrate that lentiviral vectors pseudotyped with the VSV envelope are able to transduce a hematopoietic progenitor population qualitatively different from that transduced by MLV retroviruses. Although both MLV and lentiviral vectors efficiently transduced CD34$^+$ progenitors stimulated to divide during transduction, only lentiviruses could transduce more primitive, quiescent progenitors. The most stringent test of this ability was successful transduction of ELTC-IC, a subpopulation of CD34$^+$CD38$^-$ cells that divide late in culture despite continuous cytokine stimulation. The demonstration of the transgene in CFU arising after 60 days of ELTC, and in late-appearing clones derived from single CD34$^+$CD38$^-$ cells, proved the stable integration of the lentiviral vector into CD34$^+$CD38$^-$ ELTC-IC.

Table 2. Clonal analysis of GFP transgene expression in single transduced CD34$^+$CD38$^-$ cells

| Week | MLV/GALV | MLV/VSV | lenti/VSV |
|---|---|---|---|
| 1 and 2 | 2/80 | 0/105 | 49/172 |
| 3 | 0/31 | 0/48 | 25/92 |
| 4 | 0/11 | 0/7 | 8/18 |
| 5 | 0/2 | 0/3 | 1/3 |
| Total | 2/124 (2%)* | 0/163 (0%)† | 83/285 (29%) |

Analysis of GFP expression in clones from single CD34$^+$CD38$^-$ cells grown in cobblestone area-forming cell assay. Shown is the number of GFP$^+$ colonies/the total number of colonies ($n$ = 2). *, $P$ = 0.03; and †, $P$ = 0.04 compared to lenti/VSV.

Previous reports on the transduction of human hematopoietic progenitors with lentiviral vectors have used short-term functional assays or immunophenotypic definitions as surrogate markers of HSC (25, 30). These approaches can result in misleading conclusions. Short-term assays of CD34$^+$ cells (e.g., CFU) measure mature progenitors, most of which are cycling and divide rapidly with growth factor stimulation. These cells are readily transduced by MLV vectors, and they have little or no long-term engrafting ability (6, 47–50). Although immunophenotypic definitions have been helpful for the enrichment of HSC, populations such as CD34$^+$CD38$^-$ cells are functionally heterogeneous, particularly with respect to cytokine responsiveness and their ability to be transduced (11, 51). By studying CD34$^+$CD38$^-$ cells in ELTC, we were able to measure a subpopulation of slowly dividing cells that possess other primitive characteristics expected of HSC, namely tremendous generative capacity (11) and pluripotentiality (45). It is likely that ELTC-IC are a population similar if not identical to the long-term repopulating CD34$^+$CD38$^-$ cells measured by two *in vivo* assays of human HSC, the beige–nude–xid (bnx) and non-obese-diabetic/severe combined immune deficient (NOD/SCID) xenograft models (15, 52). CD34$^+$CD38$^-$ cells that repopulate bnx and NOD/SCID mice are also highly resistant to transduction with MLV.

A second problem with using short-term assays for the assessment of stable lentiviral transduction is that transient expression can occur from nonintegrated lentiviral vectors. Pseudotransduction, particularly when using VSV pseudotyped vectors, can also result in transient detection of marker protein (37). This potential for artifact from nonintegrated lentivirus was clearly shown in our studies by short-term transgene expression in up to 38% of nondividing cells with an integrase-defective lentiviral vector, although the average expression level of the transgene was significantly lower than with wild-type vector. The integrase-defective vector was unable to produce stable long-term expression, suggesting that integration and not nuclear localization limits stable transduction of cells prior to cell division. Only integration of vectors into the target cell genome will allow the permanent and enduring clinical benefit desired in clinical trials of HSC gene therapy.

In this report we compared lentivirus with the MLV/GALV retroviral vector in all assays of transduction, as MLV has long been the gold standard in vector technology for HSC. The moi used for lenti/VSV was higher than for MLV/GALV based on titers obtained with short-term assay of 293 cells. However, pseudotransduction and/or transient expression in 293 cells may result in inaccurately high titers with lenti/VSV. It is therefore difficult to directly compare vectors based on moi. Uchida *et al.* (31) compared lentiviral vectors to MLV vectors in short-term assays and in clonal assay and showed stable integration of lentivirus into CD34$^+$Thy-1$^+$CD38$^{-/lo}$ cells. This study and our own provide the most compelling evidence to date of the superiority of lentiviral vectors pseudotyped with VSV over MLV-based vectors.

The finding that $CD34^+CD38^-$ cells can be transduced even in the absence of growth factor stimulation and after only brief exposure to lentivirus confirmed that lentiviruses can transduce primitive, quiescent progenitors. Currently, several days of *ex vivo* stimulation are required to induce cycling for successful transduction of progenitors with MLV, after which much of the long-term repopulating ability is lost (12–14, 16, 17). The ability to transduce HSC without growth factor stimulation and to minimize the time that HSC spend *ex vivo* has obvious advantages for preservation of stem cell function. Studies analyzing gene transfer into long-term repopulating cells of large animals and using xenograft models to study human long-term repopulating cells will provide further information on the advantages that lentiviruses offer for HSC transduction. Third-generation, self-inactivating HIV-1-based vectors (32) are currently under study with biosafety issues in mind. The findings presented here strongly suggest that lentiviruses may provide the technical leap needed to achieve therapeutic levels of gene transfer into human HSC and justify further intensive investigation into this vector strategy.

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# A Third-Generation Lentivirus Vector with a Conditional Packaging System

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**Vectors derived from human immunodeficiency virus (HIV) are highly efficient vehicles for in vivo gene delivery. However, their biosafety is of major concern. Here we exploit the complexity of the HIV genome to provide lentivirus vectors with novel biosafety features. In addition to the structural genes, HIV contains two regulatory genes, *tat* and *rev*, that are essential for HIV replication, and four accessory genes that encode critical virulence factors. We previously reported that the HIV type 1 accessory open reading frames are dispensable for efficient gene transduction by a lentivirus vector. We now demonstrate that the requirement for the *tat* gene can be offset by placing constitutive promoters upstream of the vector transcript. Vectors generated from constructs containing such a chimeric long terminal repeat (LTR) transduced neurons in vivo at very high efficiency, whether or not they were produced in the presence of Tat. When the *rev* gene was also deleted from the packaging construct, expression of *gag* and *pol* was strictly dependent on Rev complementation in *trans*. By the combined use of a separate nonoverlapping Rev expression plasmid and a 5′ LTR chimeric transfer construct, we achieved optimal yields of vector of high transducing efficiency (up to $10^7$ transducing units [TU]/ml and $10^4$ TU/ng of p24). This third-generation lentivirus vector uses only a fractional set of HIV genes: *gag*, *pol*, and *rev*. Moreover, the HIV-derived constructs, and any recombinant between them, are contingent on upstream elements and *trans* complementation for expression and thus are nonfunctional outside of the vector producer cells. This split-genome, conditional packaging system is based on existing viral sequences and acts as a built-in device against the generation of productive recombinants. While the actual biosafety of the vector will ultimately be proven in vivo, the improved design presented here should facilitate testing of lentivirus vectors.**

Lentiviruses have attracted the attention of gene therapy investigators (45) for their ability to integrate into nondividing cells (8, 15, 16, 25, 26). We previously developed replication-defective vectors from the lentivirus human immunodeficiency virus (HIV) and showed that they transduce target cells independent of mitosis (32). The vectors proved highly efficient for in vivo gene delivery and achieved stable long-term expression of the transgene in several target tissues, such as the brain (5, 33), the retina (31), and the liver and muscle of adult rats (21). A major concern, however, is the biosafety of vectors derived from a highly pathogenic human virus.

The complexity of the lentivirus genome may be exploited to build novel biosafety features in the design of a retrovirus vector. In addition to the structural *gag*, *pol*, and *env* genes common to all retroviruses, HIV contains two regulatory genes, *tat* and *rev*, essential for viral replication, and four accessory genes, *vif*, *vpr*, *vpu*, and *nef*, that are not crucial for viral growth in vitro but are critical for in vivo replication and pathogenesis (27).

The Tat and Rev proteins regulate the levels of HIV gene expression at transcriptional and posttranscriptional levels, respectively. Due to the weak basal transcriptional activity of the HIV long terminal repeat (LTR), expression of the provirus initially results in small amounts of multiply spliced transcripts coding for the Tat, Rev, and Nef proteins. Tat increases dramatically HIV transcription by binding to a stem-loop structure (transactivation response element [TAR]) in the nascent RNA, thereby recruiting a cyclin-kinase complex that stimulates transcriptional elongation by the polymerase II complex (46). Once Rev reaches a threshold concentration, it promotes the cytoplasmic accumulation of unspliced and singly spliced viral transcripts, leading to the production of the late viral proteins. Rev accomplishes this effect by serving as a connector between an RNA motif (the Rev-responsive element [RRE]), found in the envelope coding region of the HIV transcript, and components of the cell nuclear export machinery. Only in the presence of Tat and Rev are the HIV structural genes expressed and new viral particles produced (27).

In a first generation of HIV-derived vectors (32), viral particles were generated by expressing the HIV type 1 (HIV-1) core proteins, enzymes, and accessory factors from heterologous transcriptional signals and the envelope of another virus, most often the G protein of the vesicular stomatitis virus (VSV G) (9) from a separate plasmid. In a second version of the system, the HIV-derived packaging component was reduced to the *gag*, *pol*, *tat*, and *rev* genes of HIV-1 (51). In either case, the vector itself carried the HIV-derived *cis*-acting sequences necessary for transcription, encapsidation, reverse transcription, and integration (2, 4, 22, 24, 29, 30, 32, 35). It thus encompassed, from the 5′ to 3′ end, the HIV 5′ LTR, the leader sequence and the 5′ splice donor site, approximately 360 bp of the *gag* gene (with the *gag* reading frame closed by a synthetic stop codon), 700 bp of the *env* gene containing the RRE and a splice acceptor site, an internal promoter (typically the immediate-early enhancer/promoter of human cytomegalovirus [CMV] or that of the phosphoglycerokinase gene [PGK]), the transgene, and the HIV 3′ LTR. Vector particles are produced by cotransfection of the three constructs in 293T cells (32). In this design, significant levels of transcription from the vector

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LTR and of accumulation of unspliced genomic RNA occur only in the presence of Tat and Rev.

Here, we demonstrate that the *trans*-acting function of Tat becomes dispensable if part of the upstream LTR in the transfer vector construct is replaced by constitutively active promoter sequences. Furthermore, we show that the expression of *rev* in *trans* allows the production of high-titer HIV-derived vector stocks from a packaging construct which contains only *gag* and *pol*. This design makes the expression of the packaging functions conditional on complementation available only in producer cells. The resulting gene delivery system, which conserves only three of the nine genes of HIV-1 and relies on four separate transcriptional units for the production of transducing particles, offers significant advantages for its predicted biosafety.

## MATERIALS AND METHODS

**Transfer vector constructs.** pHR'CMV-LacZ and pHR'CMV-Luciferase have been described elsewhere (32). pHR2 is a lentivirus transfer vector in which the polylinker and downstream *nef* sequences up to the *Kpn*I site of pHR' have been replaced with a *Cla*I/*Spe*I/*Sna*BI/*Sma*I/*Bam*HI/*Sac*II/*Eco*RI polylinker. pHR2 was generated by replacing the 3.7-kb *Cla*I-*Sac*I fragment of pHR'CMVlacZ with a 607-bp *Cla*I-*Sac*I fragment generated by PCR using pHR'CMVlacZ as the template with oligonucleotide primers 5'-CCATCGATGGACTAGTCCTACGTA TCCCCGGGGACGGGATCCGCGGAATTCCGTTTAAGACCAATGAC-3' and 5'-TTATAATGTCAAGGCCTCTC-3', followed by digestion with *Cla*I and *Sac*I.

pHR2PGK-NGFR, pHR2CMV-NGFR, and pHR2MFG-NGFR are lentivirus transfer vectors in which the truncated low-affinity nerve growth factor receptor (NGFR) (6) transgenes under the control of the murine PGK, human CMV, and Moloney leukemia virus (MLV) promoters, respectively, have been inserted into the polylinker of pHR2. The pHR2PGK-NGFR transgene encodes no intron sequences, the pHR2CMV-NGFR vector includes the intron from plasmid pMD (34), and the pHR2MFG-NGFR vector contains the MLV intron from MFG-S (34).

pRRL, pRLL, pCCL, and pCLL are lentivirus transfer vectors containing chimeric Rous sarcoma virus (RSV)-HIV or CMV-HIV 5' LTRs and vector backbones in which the simian virus 40 polyadenylation and (enhancerless) origin of replication sequences have been included downstream of the HIV 3' LTR, replacing most of the human sequence remaining from the HIV integration site. In pRRL, the enhancer and promoter (nucleotides −233 to −1 relative to the transcriptional start site; GenBank accession no. J02342) from the U3 region of RSV are joined to the R region of the HIV-1 LTR. In pRLL, the RSV enhancer (nucleotides −233 to −50) sequences are joined to the promoter region (from position −78 relative to the transcriptional start site) of HIV-1. In pCCL, the enhancer and promoter (nucleotides −673 to −1 relative to the transcriptional start site; GenBank accession no. K03104) of CMV were joined to the R region of HIV-1. In pCLL, the CMV enhancer (nucleotides −673 to −220) was joined to the promoter region (position −78) of HIV-1. Exact sequences and details of construction are available on request.

pHR2hPGK-GFP, pCCLhPGK-GFP, pCLLhPGK-GFP, pRRLhPGK-GFP, and pRLLhPGK.GFP are lentivirus transfer vectors containing the enhanced green fluorescent protein (eGFP) (750-bp *Bam*HI-*Not*I fragment from pEGFP-1; Clontech) coding region, under the control of the human PGK promoter (nucleotides 5 to 516; GenBank accession no. M11958), inserted into the polylinker region of each parental vector. pRRLGFP was obtained by deletion of the *Xho*I-*Bam*HI fragment containing the PGK promoter from pRRLhPGK-GFP.

pRRLhPGK.GFP.SIN-18 is a vector in which 3' LTR sequences from −418 to −18 relative to the U3/R border have been deleted from pRLLhPGK.GFP (52).

**Packaging constructs.** The *tat*-defective packaging construct pCMVΔR8.93 was obtained by swapping an *Eco*RI-*Sac*I fragment from plasmid R7/pneo(−) (12) with the corresponding fragment of pCMVΔR8.91, a previously described plasmid expressing Gag, Pol, Tat, and Rev (51). This fragment has a deletion affecting the initiation codon of the *tat* gene and a frameshift created by the insertion of an *Mlu*I linker into the *Bsu*36I site as described previously. pCMVΔR8.74 is a derivative of pCMVΔR8.91 in which a 133-bp *Sac*II fragment, containing a splice donor site, has been deleted from the CMV-derived region upstream of the HIV sequences to optimize expression.

pMDLg/p is a CMV-driven expression plasmid that contains only the *gag* and *pol* coding sequences from HIV-1. First, *pkat*2Lg/p was constructed by ligating a 4.2-kb *Cla*I-*Eco*RI fragment from pCMVΔR8.74 with a 3.3-kb *Eco*RI-*Hind*III fragment from *pkat*2 (14) and a 0.9-kb *Hind*III-*Nco*I fragment from *pkat*2 along with an *Nco*I-*Cla*I linker consisting of synthetic oligonucleotides 5'-CATGGGT GCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGAT-3' and 5'-CG ATCTAATTCTCCCCCGCTTAATACTGACGCTCTCGCACC-3'. Next, pMDLg/p was constructed by inserting the 4.25-kb *Eco*RI fragment from *pkat*2Lg/p into the *Eco*RI site of pMD-2. pMD-2 is a derivative of pMD.G (34) in which the pXF3 plasmid backbone of pMD.G has been replaced with a minimal pUC plasmid backbone and the 1.6-kb VSV G-encoding *Eco*RI fragment has been removed.

pMDLg/pRRE differs from pMDLg/p by the addition of a 374-bp RRE-containing sequence from HIV-1 (HXB2) immediately downstream of the *pol* coding sequences. To generate pMDLg/pRRE, the 374-bp *Not*I-*Hind*III RRE-containing fragment from pHR3 was ligated into the 9.3-kb *Not*I-*Bgl*II fragment of pVL1393 (Invitrogen) along with a *Hind*III-*Bgl*II oligonucleotide linker consisting of synthetic oligonucleotides 5'-AGCTTCCGCGGA-3' and 5'-GATCTCC GCGGA-3' to generate pVL1393RRE (pHR3 was derived from pHR2 by the removal of HIV *env* coding sequences upstream of the RRE sequences in pHR2). A *Not*I site remains at the junction between the *gag* and RRE sequences. pMDLg/pRRE was then constructed by ligating the 380-bp *Eco*RI-*Sst*II fragment from pV1393RRE with the 3.15-kb *Sst*II-*Nde*I fragment from pMD-2FIX (pMD-2FIX is a human factor IX-containing variant of pMD-2 which has an *Sst*II site at the 3' end of the factor IX insert), the 2.25-kb *Nde*I-*Avr*II fragment from pMDLg/p, and the 3.09-kb *Avr*II-*Eco*RI fragment from pkat1Lg/p (14).

pRSV-Rev and pTK-Rev (generous gifts of T. Hope, Salk Institute) are *rev* cDNA-expressing plasmids in which the joined second and third exons of HIV-1 *rev* are under the transcriptional control of the RSV U3 and herpes simplex virus type 1 thymidine kinase (TK) promoters, respectively. Both expression plasmids utilize polyadenylation signal sequences from the HIV LTR in a pUC118 plasmid backbone.

**Vector production and assays.** Vectors were produced by transient transfection into 293T cells as previously described (33), with the following modifications. A total of $5 \times 10^6$ 293T cells were seeded in 10-cm-diameter dishes 24 h prior to transfection in Iscove modified Dulbecco culture medium (JRH Biosciences) with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a 5% $CO_2$ incubator, and the culture medium was changed 2 h prior to transfection. A total of 20 μg of plasmid DNA was used for the transfection of one dish: 3.5 μg of the envelope plasmid pMD.G, 6.5 μg of packaging plasmid, and 10 μg of transfer vector plasmid. The precipitate was formed by adding the plasmids to a final volume of 450 μl of 0.1× TE (1× TE is 10 mM Tris [pH 8.0] plus 1 mM EDTA) and 50 μl of 2.5 M $CaCl_2$, mixing well, then adding dropwise 500 μl of 2× HEPES-buffered saline (281 mM NaCl, 100 mM HEPES, 1.5 mM $Na_2HPO_4$ [pH 7.12]) while vortexing and immediately adding the precipitate to the cultures. The medium (10 ml) was replaced after 14 to 16 h; the conditioned medium was collected after another 24 h, cleared by low-speed centrifugation, and filtered through 0.22-μm-pore-size cellulose acetate filters. For in vitro experiments, serial dilutions of freshly harvested conditioned medium were used to infect $10^5$ cells in a six-well plate in the presence of Polybrene (8 μg/ml). Viral p24 antigen concentration was determined by immunocapture (Alliance; DuPont-NEN). Vector batches were tested for the absence of replication-competent virus by monitoring p24 antigen expression in the culture medium of transduced SupT1 lymphocytes for 3 weeks. In all cases tested, p24 was undetectable (detection limit, 3 pg/ml) once the input antigen had been eliminated from the culture. Transducing activity was expressed in transducing units (TU).

**Northern blot analysis.** Total RNA was isolated from $1 \times 10^7$ to $2 \times 10^7$ cells harvested at confluence by using RNAsol B as suggested by the manufacturer; 10 to 20 μg of RNA was loaded per well on 1% agarose gels, using NorthernMax (Ambion, Austin, Tex.) reagents as described by the manufacturer. Transfer was to Zetabind membranes (Cuno Inc., Meridien, Conn.) by either capillary transfer or pressure blotting (Stratagene). $^{32}P$-labeled probes were made by random priming.

**Intracerebral injection of vectors.** Twelve Fischer 344 male rats weighing approximately 220 g were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.). The rats were housed with access to ad libitum food and water on a 12-h light/dark cycle and were maintained and treated in accordance with published National Institutes of Health guidelines. All surgical procedures were performed with the rats under isoflurane gas anesthesia, using aseptic procedures. After a rat was anesthetized in a sleep box, it was placed in a small animal stereotaxic device (Kopf Instruments, Tujunga, Calif.) using the earbars, which do not break the tympanic membrane. The rats were randomly divided into one control and four treatment groups. After the rats were placed in the stereotaxic frame, 2 μl of lentivirus vector concentrated by ultracentrifugation at 50,000 × *g* for 140 min at 20°C (33) in phosphate-buffered saline (PBS) was injected consecutively into the striatum in both hemispheres over 4 min at a rate of 0.5 μl/min (coordinates, AP 0.0, LAT ±3.0, DV −5.5, −4.5, −3.5 with the incisor bar set at 3.3 mm below the intra-aural line [36]), using a continuous-infusion system as described previously in detail (28). During the injection, the needle was slowly raised 1 mm in the dorsal direction every 40 s (3-mm total withdrawal). One minute after cessation of the injection, the needle was retracted an additional 1 mm and then left in place for an additional 4 min before being slowly withdrawn from the brain.

**Histology.** One month after vector injection, each animal was deeply anesthetized with intraperitoneal pentobarbital and perfused through the aorta with sterile PBS, followed by ice-cold 4% paraformaldehyde perfusion. The brains were removed from the skulls, postfixed in 4% paraformaldehyde by immersion for 24 h, and then transferred into a 30% sucrose–PBS solution for 3 to 4 days, until the brains sank to the bottom of their containers. The brains were then frozen on dry ice, and 40-μm-thick coronal sections were cut on a sliding microtome. Sections were collected in series in microtiter well plates that contained a glycerin-based antifreeze solution, and they were kept at −30°C until further processing. Immunocytochemistry was performed according to the general pro-

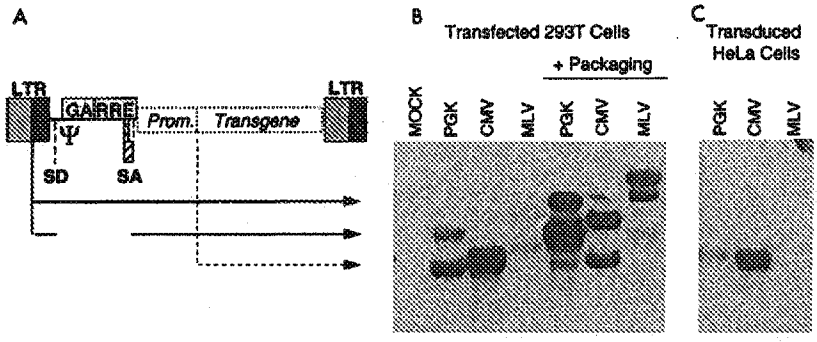


FIG. 1. Northern analysis of the RNA expression from lentivirus vectors. Three pHR2 vectors carrying an expression cassette for the same transgene (truncated low-affinity NGFR) and driven by three different promoters (PGK, CMV, and retroviral MFG) were analyzed in producer and transduced cells. Total RNA was extracted and analyzed by Northern blotting with a probe specific for the transgene sequence. (A) Schematic of the vector construct depicts the species of RNA driven by the internal promoter (Prom.; broken arrow, shorter transcript) and the viral LTR (solid arrows, longer transcripts; the two species differ for the splicing of the viral intron). The splice donor and acceptor sites (SD and SA), the packaging sequence (Ψ), the truncated *gag* sequence (GA), and the RRE are indicated. (B) The vector constructs were transfected in 293T cells without or with the packaging construct. (C) Vector particles produced by the 293T transfectants were used to transduce HeLa cells. In the absence of the viral transactivators, supplied by the core packaging construct only in the producer cells, vector expression occurs mainly from the internal promoter. Note the dramatic enhancement of the upstream transcription and the accumulation of unspliced RNA (carrying the Ψ sequence) in the presence of the packaging construct. In the transduced cells, the LTR is silenced. Note that the three expression cassettes differ in the size of the promoters and 5′ untranslated sequence. In each case, the smallest RNA species represents transcripts initiated from the internal promoter, while the intermediate-size and larger species correspond to spliced and unspliced LTR-driven RNAs, respectively.

cedure described previously (44). After several PBS rinses and an incubation in 3% hydrogen peroxide, the sections were placed in a 3% normal goat serum. The sections were then incubated in the primary anti-GFP antibody (1:1,000; Clontech, Palo Alto, Calif.) in 1% normal goat serum–0.1% Triton X-100 overnight at room temperature. After rinsing, the sections were incubated in the biotinylated rabbit anti-goat secondary antibody (Vector, Burlingame, Calif.) for 3 h. After rinsing, the sections were incubated with horseradish peroxidase-streptavidin and then reacted by using a purple chromagen kit (VIP; Vector), mounted, dried, dehydrated, and coverslipped.

## RESULTS

**Tat is required to produce a vector of efficient transducing activity.** To investigate the role of Tat in the production of transducing particles, expression from lentivirus vectors was first examined by Northern analysis (Fig. 1). The patterns of RNAs induced by transfer vectors in which the transgene was driven by an internal PGK, CMV, or retrovirus MFG promoter were studied in both producer and target cells. In transfected 293T cells, expression occurred mainly from the internal promoter. When a packaging construct expressing both Tat and Rev was cotransfected, a dramatic enhancement of transcription from the LTR was observed, with an accumulation of unspliced vector RNA. In cells transduced with the vectors, that is, in the absence of Tat and Rev, transcription from the LTR was almost completely suppressed, the residual transcripts underwent splicing, and the internal promoter was responsible for most of the expression.

A packaging plasmid carrying two mutations in *tat* (pCMVΔR 8.93) was then constructed. The first mutation is a deletion of the T in the ATG initiation codon of the *tat* gene; the second is an insertion of a *Mlu*I linker producing a translation stop codon after residue 46 of the Tat protein. These changes confer a *tat*-defective phenotype to HIV-1 (12). After transfection of the control or *tat*-defective packaging constructs into 293T cells, comparable yields of vector particles were recovered in the culture medium, as assayed by using the Gag p24 antigen (see Table 3). Such Tat independence was expected from the replacement of the HIV LTR by the constitutive CMV promoter in the packaging construct. However, the particles produced in the absence of Tat had a dramatically reduced transducing activity (Table 1): 5 to 15% of that of particles produced by the control Tat-positive packaging construct.

We also tested whether the Tat-defective phenotype could be rescued by complementation in target cells (Table 1). HeLa-tat cells, a cell line expressing Tat from the HIV-1 LTR (13), were transduced by vectors produced with or without Tat. The expression of Tat in target cells did not compensate for the loss in transduction efficiency of vector produced without Tat.

As expected from the Northern analysis, functional inactivation of the *tat* gene resulted in a lower abundance of vector RNA in producer cells. This was indicated by the decrease in luciferase activity in cells producing a luciferase vector without an internal promoter. In this case, transgene expression di-

TABLE 1. Transducing activities of lentivirus vectors made with and without a functional *tat* gene in the packaging construct[a]

| Transfer vector | Target cells | Mean transducing activity (TU/ng of p24) ± SE[b] | |
|---|---|---|---|
| | | With *tat* in packaging construct | Without *tat* in packaging construct |
| pHR′CMV-LacZ | 293T | 1,056 ± 54 | 152 ± 26 |
| pHR2PGK-eGFP | HeLa | 5,666 | 384 |
| pHR′CMV-Luciferase | HeLa | 3,000 ± 152 | 152 ± 26 |
| | HeLa-tat | 3,777 ± 348 | 486 ± 59 |
| pHR′Luciferase[c] | HeLa | 46 ± 1 | 0.3 ± 0.003 |
| | HeLa-tat | 3,296 ± 276 | 174 ± 75 |

[a] Vectors were produced by transfection of the indicated transfer vector, a packaging construct either with (pCMVΔR8.91) or without (pCMVΔR8.93) a functional *tat* gene, and plasmid pMD.G into 293T cells. Serial dilutions of transfectant conditioned medium were incubated with the indicated cells, and the cultures were scored after 3 days. For calculating transduction activity, samples were selected from the linear portion of the vector dose-response curve.

[b] LacZ transduction was measured by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining and by expression of the number of blue cell colonies as a function of the amount of p24 antigen in the inoculum. eGFP transduction was measured by FACS analysis, multiplying the fraction of fluorescent cells by the number of infected cells, and expressing the result as a function of the amount of p24 antigen in the inoculum. Luciferase transduction was measured by luminescence in RLU above background of 50 μl of culture extract and dividing the number of RLU × $10^{-2}$ by the number of nanograms of p24 antigen in the inoculum. Means of duplicate (pHR2 PGK-eGFP) or triplicate (all other constructs) determinations are shown.

[c] Without internal promoter.

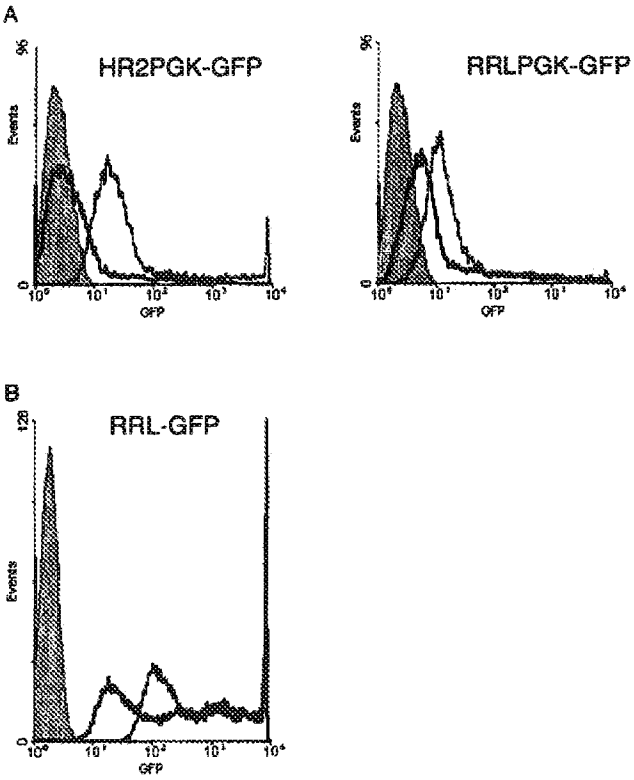


FIG. 2. Transcriptional activities of wild-type and 5′ chimeric vector constructs in the absence and presence of Tat. (A) Control pHR2 and the 5′ chimeric pRRL transfer construct carrying a PGK-eGFP expression cassette were transfected into 293T cells with a packaging construct having a functional (pCMVΔR8.91; grey line) or inactive (pCMVΔR8.93; black line) *tat* gene. GFP expression was analyzed by FACS. The filled area represents nontransfected cells. In the absence of Tat, the chimeric construct yielded a level of GFP expression higher than that achieved by the pHR2 construct. Both constructs were further upregulated by Tat. (B) A pRRL construct carrying the eGFP gene without an internal promoter was transfected with a packaging construct carrying a functional (grey line, open area) or inactive (black line, open area) *tat* gene. Direct upregulation of the chimeric promoter by Tat was observed. The filled area represents nontransfected cells.

rectly reflects the abundance of transcripts originating from the LTR. 293T cells producing luciferase vectors without Tat had only 5% of the luciferase content of cells producing the same vector with Tat ($[1.0 \pm 0.2] \times 10^9$ relative light units [RLU]/dish without Tat; $[20.2 \pm 0.7] \times 10^9$ RLU/dish with Tat). This ratio corresponded very closely to that observed in cells transduced by either type of vector in the course of the same experiment (Table 1), suggesting that the abundance of vector RNA in producer cells is a rate-limiting factor in the transduction by lentivirus vectors.

One could thus conclude that Tat is required in producer cells to activate transcription from the HIV LTR and to generate vector particles with a high transducing activity.

**The *tat* requirement is offset by placing a constitutive promoter upstream of the transfer vector.** If the only function of Tat is *trans* activation of vector transcription from the LTR, the *tat*-defective phenotype should be rescued by placing a strong constitutive promoter upstream of the vector transcript. Three transcriptional domains have been identified in the HIV promoter in the U3 region of the LTR: the core or basal domain, the enhancer, and the modulatory domain (27). Transcription starts at the U3/R boundary, the first nucleotide of R being numbered 1. The core promoter contains binding sites for the TATA-binding protein (−28 to −24) and SP-1 (three binding sites between −78 to −45). The enhancer contains two binding sites for NF-κB which overlap with a binding site for NFATc (−104 to −81). The modulatory domain contain binding sites for several cellular factors, including AP-1 (−350 to −293), NFAT-1 (−256 to −218), USF-1 (−166 to −161), Ets-1 (−149 to −141), and LEF (−136 to −125). A panel of 5′ chimeric transfer constructs carrying substitutions of either all or part of the U3 region of the 5′ LTR was generated. All substitutions were made to preserve the transcription initiation site of HIV. Partial substitutions joined new enhancer sequences to the core promoter of the HIV LTR (−78 to 1), while full substitutions replaced also the promoter. pRLL and pRRL vectors carried the enhancer and the enhancer/promoter, respectively, of RSV; pCLL and pCCL vectors carried the enhancer and the enhancer/promoter of human CMV.

Control pHR2 and 5′ chimeric transfer constructs carrying a PGK-eGFP expression cassette were tested by transfection of 293T cells with control or *tat*-defective packaging constructs, and the expression of the eGFP transgene was analyzed by fluorescence-activated cell sorting (FACS). The RRL chimeric construct yielded a higher level of eGFP expression than the pHR2 vector, reflecting the constitutive transcriptional activity of the new sequence (Fig. 2A). Interestingly, the chimeric vector also displayed upregulation by Tat, as shown by the increased eGFP expression of cells cotransfected with the control packaging construct. Tat upregulation was proven to be a direct effect by transfecting a pRRL-eGFP vector lacking an internal promoter with control or *tat*-defective packaging constructs and analyzing GFP expression by FACS (Fig. 2B). Comparable results were obtained with the other chimeric LTR vectors (not illustrated). Vector particles were then collected from the transfected producer cells and assayed for transduction of eGFP into HeLa cells and human primary lymphocytes (peripheral blood lymphocytes [PBL]). As shown in Table 2, all vectors had efficient transducing activity, as assessed by endpoint titration on HeLa cells or maximal transduction frequency of PBL. The vector produced by the pRRL chimera was as efficient as that produced by the pHR2 construct and was selected to test transduction independent of Tat. As shown in Table 3, the pRRL construct yielded a vector of only slightly reduced transducing activity (60%) when the packaging construct was *tat* defective. The residual effect of

TABLE 2. GFP transduction by lentivirus vectors made by transfer constructs with a wild-type or 5′ chimeric LTR

| Transfer construct | Endpoint titer on HeLa cells (TU/ml)[a] | Transduction efficiency on human lymphocytes (% positive cells)[b] |
|---|---|---|
| pHR2 | $2.3 \times 10^7$ | 30 |
| pCCL | $4.6 \times 10^6$ | 14 |
| pCLL | $7.9 \times 10^6$ | 18 |
| pRRL | $1.8 \times 10^7$ | 29 |
| pRLL | $8.9 \times 10^6$ | 18 |

[a] Determined by multiplying the percentage of fluorescent cells for the vector dilution and the number of infected cells. Samples were selected from the linear portion of the vector dose-response curve.

[b] Percentage of fluorescent human PBL after infection of $10^6$ cells with 1 ml of vector containing medium. Primary human T lymphocytes were isolated and transduced as previously described (14). Vectors carrying a PGK-eGFP expression cassette were produced by transfection of the indicated transfer construct, the packaging plasmid pCMVΔR8.91, and the envelope plasmid pMD.G into 293T cells. Fluorescent cells were scored by FACS analysis 6 days after transduction. Data are averages of duplicate determinations for a representative experiment of three performed.

TABLE 3. GFP transduction into HeLa cells by lentivirus vectors made by transfer constructs with a wild-type or 5′ chimeric LTR and packaging constructs with or without a functional *tat* gene[a]

| Transfer construct | *tat* gene in packaging construct | Endpoint titer (TU/ml) | p24 antigen (ng/ml) | Transduction efficiency (TU/ng of p24) |
|---|---|---|---|---|
| pHR2 | + | $4.1 \times 10^6$ | 297 | 13,805 |
| pHR2 | − | $2.4 \times 10^5$ | 545 | 440 |
| pRRL | + | $1.3 \times 10^7$ | 546 | 23,810 |
| pRRL | − | $4.9 \times 10^6$ | 344 | 14,244 |

[a] Vectors carrying a PGK-eGFP expression cassette were produced by transfection of the indicated transfer and packaging plasmid plus plasmid pMD.G into 293T cells. Serial dilutions of transfectant conditioned medium were incubated with HeLa cells, and the cultures were scored after 6 days. For calculating endpoint titers, samples were selected from the linear portion of the vector dose-response curve. Data are averages of duplicate determinations for a representative experiment of five performed.

Tat on transduction was in agreement with the ability of Tat to upregulate transcription from the chimeric LTR.

The use of the chimeric LTR construct allowed removal of Tat from the packaging system with a minimal loss in the transduction efficiency of the vector in vitro. To test vector performance in the more challenging setting of in vivo delivery into brain neurons, high-titer vector stocks were generated from the pHR2 and pRRL constructs with and without Tat. The four stocks of eGFP vector were matched for particle content by p24 antigen and injected bilaterally in the neostriata of groups of three adult rats. The animals were sacrificed after 1 month, and serial sections of the brain were analyzed for eGFP fluorescence (not shown) and immunostained by antibodies against eGFP (Fig. 3). The results obtained in vivo matched the in vitro data. Vector produced by the pHR2 construct only achieved significant transduction of the neurons when packaged in the presence of Tat. Vector produced by the pRRL chimera was as efficient when made with or without Tat. The transduction extended throughout most of the striatum and reached a very high density of positive cells in the sections closest to the injection site. No signs of pathology were detectable in the injected tissue, except for a small linear scar marking the needle track, by hematoxylin and eosin staining of the sections (data not shown).

These results provide evidence that Tat is dispensable for efficient transduction by a lentivirus vector.

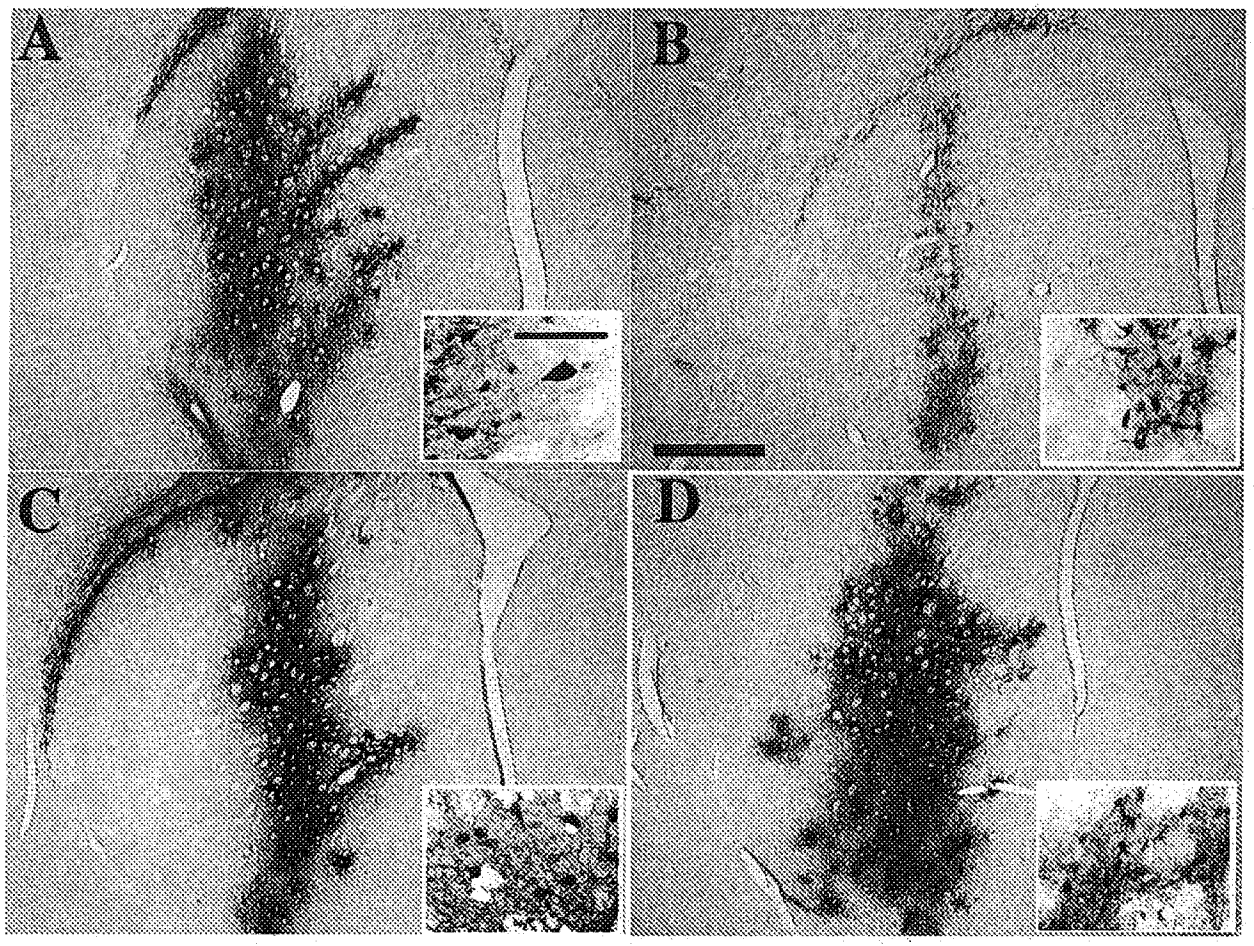


FIG. 3. In vivo transduction of eGFP into brain cells by lentivirus vectors produced with and without Tat. Vectors carrying a PGK-eGFP expression cassette were produced by the pHR2 (A and B) or the 5′ chimeric pRRL (C and D) transfer construct and a packaging construct with (pCMVΔR8.91; A and C) or without (pCMVΔR8.93; B and D) a functional *tat* gene, concentrated by ultracentrifugation, and normalized for particle content prior to injection into the corpora striata of adult rats. One month after injection, brain sections were stained for immunoreactivity to the GFP protein. While both types of vectors transduced neurons very efficiently when made with Tat, only the vector made by the chimeric transfer construct worked as well when produced without Tat. Representative sections close to the injection site are shown for one of six striata injected per each type of vector. The bar in panel B represents 1 mm; that in the inset in panel A represents 100 μm.

TABLE 4. GFP transduction into HeLa cells by lentivirus vectors made by linked or split packaging constructs and a pRRL transfer construct[a]

| Packaging construct | Separate *rev* plasmid[b] | p24 antigen (ng/ml) | Endpoint titer (TU/ml) | Transduction efficiency (TU/ng of p24) |
|---|---|---|---|---|
| pCMVΔR8.74 | | 364 | $1.07 \times 10^7$ | 29,436 |
| pMDLg/pRRE | | <0.1 | ND | NA |
| pMDLg/pRRE | TK-Rev, 5 μg | 29 | $6.9 \times 10^5$ | 23,793 |
| pMDLg/pRRE | TK-Rev, 12 μg | 94 | $2.02 \times 10^6$ | 21,489 |
| pMDLg/pRRE | RSV-Rev, 2.5 μg | 774 | $1.0 \times 10^7$ | 13,495 |
| pMDLg/pRRE | RSV-Rev, 5 μg | 776 | $7.6 \times 10^6$ | 9,761 |
| pMDLg/pRRE | RSV-Rev, 12 μg | 565 | $4.8 \times 10^6$ | 8,495 |

[a] Vectors carrying a PGK-eGFP expression cassette were produced by the transfection of a self-inactivating pRRL transfer construct (with a deletion in the 3′ LTR [53]), the indicated packaging and *rev* plasmids, and plasmid pMD.G into 293T cells. Serial dilutions of transfectant conditioned medium were incubated with HeLa cells, and the cultures were scored after 6 days. For calculating endpoint titers, samples were selected from the linear portion of the vector dose-response curve. Data are averages of duplicate determination for a representative experiment of three performed. ND, none detected (the detection limit of the assay was $10^2$ TU/ml); NA, not applicable.

[b] The promoter driving the expression of a synthetic *rev* cDNA and the amount of plasmid transfected are indicated.

**A new split-genome conditional packaging system.** The possibility of deleting the *tat* gene prompted us to explore a new design of the packaging component of the HIV vector system, in which two separate nonoverlapping expression plasmids, one for the *gag* and *pol* genes and the other for the *rev* gene, were used. The *gag* and *pol* reading frames were expressed within the context of the MD cassette, which employs the CMV promoter and intervening sequence and the human β-globin poly(A) site (34). All HIV sequences upstream of the *gag* initiation codon were removed, and the leader was modified for optimal fit to the Kozak consensus for translation. This construct, however, expressed almost no p24 antigen when transfected alone in 293T cells. This observation is in agreement with the previously reported presence of *cis*-repressive or inhibitory sequences in the *gag* and *pol* genes (40, 41). The HIV RRE was then inserted downstream of the *pol* gene, and the resulting plasmid was cotransfected with a *rev* expression vector (Table 4). High levels of p24 antigen production were observed in this case, the highest yields being obtained when *rev* was driven by an RSV promoter. When the *gag-pol* and the *rev* constructs were cotransfected with the pRRL chimeric transfer vector and the VSV G-expressing plasmid, high-titer vector was obtained in the culture medium. Both the yield of particles and their transducing efficiency were similar to those obtained with previous versions of the system. Northern analysis of producer cells confirmed that unspliced vector genomic RNA accumulated only in the presence of Rev (data not shown). Thus, both the expression of the *gag* and *pol* genes and the accumulation of packageable vector transcripts are dependent on *trans* complementation by a separate Rev expression construct. Such a conditional packaging system provides an important safety feature unavailable to oncoretrovirus vectors.

## DISCUSSION

The predicted biosafety of a viral vector depends in part on how much segregation of the *cis*- and *trans*-acting functions of the viral genome is achieved by the vector design and is maintained during vector production. A vector particle is assembled by viral proteins expressed in the producer cell from a construct(s) stripped of the *cis*-acting sequences required for the transfer of the viral genome to target cells (packaging construct). These *cis*-acting sequences are instead linked to the transgene in the transfer vector. As the vector particle packages only the genetic information contained in this latter construct, the infection process is limited to a single round without spreading. Through recombination, it is possible that sequences encoding viral proteins rejoin the *cis*-acting elements of the transfer vector. If the resulting recombinant expresses all required functions, it is able to replicate (i.e., it is a replication-competent retrovirus [RCR]) and presents a risk to the recipient. The formation of heterozygous vector particles containing RNAs from both the packaging and transfer vectors, followed by homologous recombination during reverse transcription, is the mechanism most often incriminated in the emergence of RCR during the production of retroviral vectors. The likelihood of this type of recombination is dependent on residual *cis*-acting sequences in the packaging plasmid, allowing some level of encapsidation, and on the extent of homology between packaging and vector constructs (10).

A first strategy to improve the biosafety of a vector is to use nonoverlapping split-genome packaging constructs that require multiple recombination events with the transfer vector for RCR generation. Earlier studies described several approaches to generate replication-defective HIV vectors (7, 35, 38, 42). However, these vectors could be produced only to low infectious titers, were restricted to CD4-positive cellular targets, and carried the risk of generating wild-type HIV by recombination of the components. A major advance was achieved when an improved vector design was combined with the use of the envelope of another virus (32, 33, 39). The lentivirus vector that we describe here is packaged by three nonoverlapping expression constructs, two expressing HIV proteins and the other expressing the envelope of a different virus. Moreover, all HIV sequences known to be required for encapsidation and reverse transcription (2, 22, 24, 27, 29, 30, 35) are absent from these constructs, with the exception of the portion of the *gag* gene that contributes to the stem-loop structure of the HIV-1 packaging motif (29).

A second strategy to improve vector biosafety took advantage of the complexity of the lentivirus genome. The minimal set of HIV-1 genes required to generate an efficient vector was identified, and all other HIV reading frames were eliminated from the system. As the products of the removed genes are important for the completion of the virus life cycle and for pathogenesis, no recombinant can acquire the pathogenetic features of the parental virus. We previously demonstrated that all four accessory genes of HIV could be deleted from the packaging construct without compromising gene transduction (51). In this work, we went further by deleting another factor crucial for HIV replication, the *tat* gene. Its product is one of the most powerful transcriptional activators known and plays a pivotal role in the exceedingly high replication rates that characterize HIV-induced disease (18, 19, 47).

It was found that Tat was required in producer cells to generate vector of efficient transducing activity but that this requirement was offset by inducing constitutive high-level expression of vector RNA. Due to the low basal transcription from the HIV LTR, Tat was necessary to increase the abundance of vector transcripts and allow their efficient encapsidation by the vector particles. When made in the absence of Tat, vector particles had 10- to 20-fold-reduced transducing activity. However, when strong constitutive promoters replaced the HIV sequence in the 5′ LTR of the transfer construct, vectors made without Tat exhibited a less than twofold reduction in transducing activity. As Tat strongly upregulated transcription from the chimeric LTR, the transducing activity of the output particles must reach saturation. The abundance of vector RNA

in producer cells thus appears to be a rate-limiting factor for transduction until it reaches a threshold. Conceivably, an upper limit is set by the total output of particles available to encapsidate vector RNA. As the total particle output varied with the types of vector and internal promoter used, this may explain the quantitative differences obtained in response to *tat* deletion.

Successful deletion of the *tat* gene was unexpected in view of a reported additional role for Tat in reverse transcription (17, 20). While the reasons for this discrepancy are not obvious, it should be noted that the transduction pathway of the lentivirus vector mimics only in part the infection pathway of HIV. The vector is pseudotyped by the envelope of an unrelated virus and contains only the core proteins of HIV, without any accessory gene product. The VSV envelope targets the vector to the endocytic pathway, and it has been shown that redirection of HIV-1 from its normal route of entry by fusion at the plasma membrane significantly changes the biology of the infection. For example, Nef and cyclophilin A are required for the optimal infectivity of wild-type HIV-1 but not of a (VSV G) HIV pseudotype (1). It is also possible that the kinetics of reverse transcription are more critical for the establishment of viral infection than for gene transduction, given the differences in size and sequence between the virus and vector genome.

Tat-independent transduction by an HIV-based vector was recently reported by Kim et al. for in vitro cellular targets (23). In the vector designed by these authors, however, Tat and Rev were expressed from the transfer vector and thus were also present in target cells. A CMV-HIV hybrid LTR was used; this construct yielded vector titers approximately 30% of that obtained with an intact LTR. When the *tat* gene was inactivated, the titer did not change. Srinivasakumar et al. (43) previously reported a rather low (5- to 10-fold) dependence on Tat of an HIV-based vector produced by cells stably expressing the HIV structural proteins. In this case, titers of $5 \times 10^3$ TU/ml with Tat and $7 \times 10^2$ TU/ml without Tat were obtained on HeLa-CD4 cells. Although these titers are much lower than those reported here, the vector particles carried the HIV envelope, an indication that Tat is not absolutely required for transduction by vector particles which in that case mirror more closely the wild-type virus. It remained possible, however, that a dependence on Tat may be revealed in more challenging gene deliveries into the body tissues that are the actual targets of gene therapy. This could have been due to a stricter Tat requirement for optimal transduction efficiency or for the production of high-titer vector stocks or to differences in cell-type-specific factors. Our results now establish that Tat is fully dispensable for lentivirus vector transduction even when high titers are achieved and, most importantly, for gene delivery in vivo into terminally differentiated neurons of an adult rat brain.

The Northern analysis of producer and target cells shows that the Tat dependence of LTR-driven expression restricts the production of vector genomic RNA to producer cells. This applies as well to vectors made by the 5′ chimeric constructs, as the U3 sequences of both LTRs of the resulting provirus are derived from the vector 3′ LTR. However, the functional replacement of the *tat* gene in the packaging construct by promoter sequences upstream of the transfer construct makes the generation of a transcriptionally active recombinant much more unlikely. This will be even more significant in stable producer cell lines that avoid the risk of plasmid recombination during cotransfection.

We also exploited the Rev dependence of *gag-pol* expression and of the accumulation of unspliced, packageable transcripts. Yu et al. (50) previously showed that the dependence on Rev can be used to make expression of HIV genes inducible. We

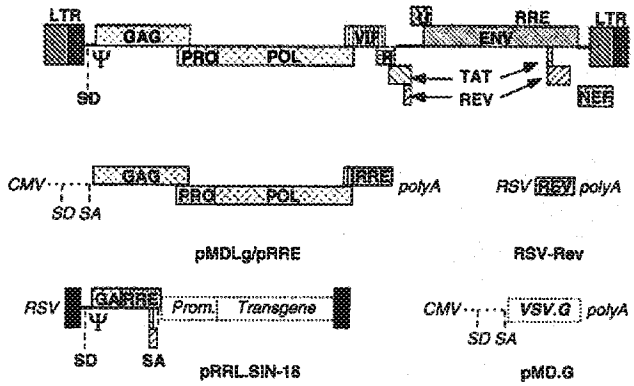


FIG. 4. Schematic drawing of the HIV provirus and the four constructs used to make a lentivirus vector of the third generation. The viral LTRs, the reading frames of the viral genes, the major 5′ splice donor site (SD), the packaging sequence (Ψ), and the RRE are boxed and indicated in bold type. The conditional packaging construct, pMDLg/pRRE, expresses the *gag* and *pol* genes from the CMV promoter and intervening sequences and polyadenylation site of the human β-globin gene. As the transcripts of the *gag* and *pol* genes contain *cis*-repressive sequences, they are expressed only if Rev promotes their nuclear export by binding to the RRE. All *tat* and *rev* exons have been deleted, and the viral sequences upstream of the *gag* gene have been replaced. A nonoverlapping construct, RSV-Rev, expresses the *rev* cDNA. The transfer construct, pRRL.SIN-18, contains HIV-1 *cis*-acting sequences and an expression cassette for the transgene. It is the only portion transferred to the target cells and does not contain wild-type copies of the HIV LTR. The 5′ LTR is chimeric, with the enhancer/promoter of RSV replacing the U3 region (RRL) to rescue the transcriptional dependence on Tat. The 3′ LTR has an almost complete deletion of the U3 region, which includes the TATA box (from nucleotides −418 to −18 relative to the U3/R border). As the latter is the template used to generate both copies of the LTR in the integrated provirus, transduction of this vector results in transcriptional inactivation of both LTRs; thus, it is a self-inactivating vector (SIN-18). The fourth construct, pMD.G, encodes a heterologous envelope to pseudotype the vector, here shown coding for VSV G. Only the relevant parts of the constructs are shown.

describe a core packaging system split in two separate nonoverlapping expression constructs, one for the *gag* and *pol* reading frames optimized for Rev-dependent expression and the other for the *rev* cDNA. This third-generation packaging system matches the performance of its predecessors in terms of both yield and transducing efficiency. However, it increases significantly the predicted biosafety of the vector. It has been suggested that the Rev-RRE axis could be replaced by the use of constitutive RNA transport elements of other viruses, although at the price of decreased efficiency (11, 23, 43). We would suggest that maintaining the Rev dependence of the system allows for an additional level of biosafety through the splitting of the HIV-derived components of the packaging system.

The conditional packaging system described here can be combined with a self-inactivating vector construct carrying a major deletion in the 3′ LTR (52). This vector design (Fig. 4) offers significant biosafety features. The contribution of HIV is reduced to a fraction of *cis*-acting sequences in the vector, leaving out in particular most of the LTR, and to only three genes, *gag*, *pol*, and *rev*, in the packaging constructs, compared with the nine genes necessary for the in vivo replication and pathogenesis of wild-type HIV-1 (3, 18, 27, 49). The actual biosafety of a vector must be proven in vivo. However, given the serious limitations of the available animal models of HIV-induced disease, the biosafety of HIV-derived vectors will ultimately be proven only in human hosts. Therefore, the vector design must ensure the highest predictable biosafety for clinical testing to be acceptable.

It is noteworthy that the fraction of the HIV-1 genome that is left in the vector is probably smaller than could be achieved

with any of the nonprimate lentiviruses, the genomic complexity of which is lower than that of HIV-1 (37). Also, the risks associated with the introduction in humans of a recombinant arising from a nonprimate lentivirus, even in a form that in its cognate animal species appears to be attenuated, are very difficult to assess, as illustrated by the ongoing debate on xenotransplantation (48). In contrast, the almost two decades spent studying a virus that has now spread in tens of millions of people worldwide have revealed a considerable amount of information on the pathogenic features of HIV-1, in particular on the dependence of virulence on a crucial set of viral genes. Based on these data, we would like to suggest that the HIV-based vectors described here are good candidates for the clinical trial of lentivirus vectors in human gene therapy.

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# In Vivo Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector

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A retroviral vector system based on the human immunodeficiency virus (HIV) was developed that, in contrast to a murine leukemia virus–based counterpart, transduced heterologous sequences into HeLa cells and rat fibroblasts blocked in the cell cycle, as well as into human primary macrophages. Additionally, the HIV vector could mediate stable in vivo gene transfer into terminally differentiated neurons. The ability of HIV-based viral vectors to deliver genes in vivo into nondividing cells could increase the applicability of retroviral vectors in human gene therapy.

Until now, gene therapy protocols have often relied on vectors derived from retroviruses such as murine leukemia virus (MLV) (*1*, *2*). These vectors are useful because the genes they transduce are integrated into the genome of the target cells, a desirable feature for long-term expression. However, these retroviral vectors can only transduce dividing cells, which limits their use for in vivo gene transfer in nonproliferating cells such as hepatocytes, myofibers, hematopoietic stem cells, and neurons (*3*, *4*). The optimal gene transfer system would include a retroviral vector based on a virus, such as HIV and other lentiviruses, that can integrate into the genome of nonproliferating cells. In vitro, HIV can infect primary cultures of monocyte-derived macrophages (*5*) as well as cell cycle–arrested $CD4^+$ HeLa or T lymphoid cells (*6*). Central to this ability are karyophilic determinants contained in two virion proteins, matrix (MA) and Vpr. These proteins interact with the nuclear import machinery and mediate the active transport of the HIV preintegration complex through the nucleopore (*7*–*9*).

A three-plasmid expression system was used to generate HIV-derived retroviral vector particles by transient transfection, as described for other vectors (*10*) (Fig. 1). Plasmid pCMVΔR9, the packaging construct, contains the human cytomegalovirus (hCMV) immediate early promoter, which drives the expression of all viral proteins required in trans. This plasmid is defective for the production of the viral envelope and the accessory protein Vpu. The packaging signal (Ψ) and adjacent sequences were deleted from the 5′ untranslated region, but the 5′ splice donor site was preserved. A polyadenylation [poly(A)] site from the insulin gene was substituted for the 3′ long terminal repeat (LTR) at the end of the *nef* reading frame (*11*). This design eliminated cis-acting sequences crucial for packaging, reverse transcription, and integration of transcripts derived from the packaging plasmid (*12*). To broaden the tropism of the vector, we used a second plasmid that encodes a heterologous envelope protein for pseudotyping the particles generated by pCMVΔR9 (*13*). Two variants of this construct were used: One variant encodes the amphotropic envelope of MLV (Ampho), and the other encodes the G glycoprotein of vesicular stomatitis virus (VSV G) (*14*). The latter envelope offers the additional advantage of high stability, which allows for particle concentration by ultracentrifugation (*15*). The third plasmid, the transducing vector (pHR′), contains cis-acting sequences of HIV required for packaging, reverse transcription, and integration, as well as unique restriction sites for the cloning of heterologous complementary DNAs (cDNAs). Nearly 350 base pairs of *gag* as well as *env* sequences encompassing the Rev response element (RRE) flanked by splice signals were included in the pHR′ vector (*16*). This design had a dual purpose: first, to increase packaging efficiency, as both *gag* and *env* RNA determinants have been demonstrated to enhance this process (*17*), and second, to allow the efficient transcription and cytoplasmic export of full-length vector transcripts only in the presence of the HIV Tat and Rev regulatory proteins, both of which are encoded by the packaging plasmid, pCMVΔR9. In the absence of these transacting factors, the only detectable expression originated from the internal promoter in the vector (*18*). The *Escherichia coli* β-galactosidase (β-gal) or the firefly luciferase coding sequences were inserted into pHR′ downstream of the hCMV immediate early promoter to serve as reporter genes.

Replication-defective retroviral particles were generated by transient cotransfection of 293T human kidney cells with the three-plasmid combination (*19*). MLV-derived packaging and transducing vectors served as controls (*20*). Media from the various transfectants were first

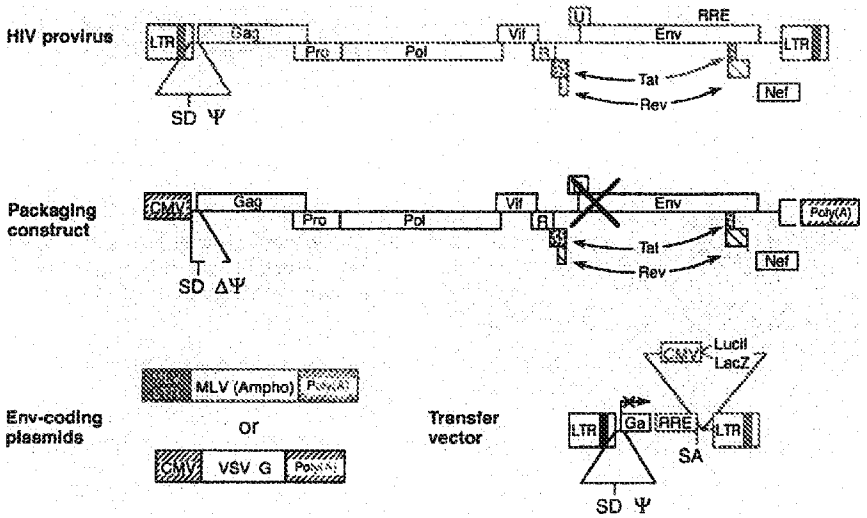


**Fig. 1.** Schematic representation of the HIV provirus and the three-plasmid expression system used for generating a pseudotyped HIV-based vector by transient transfection. Only the relevant portion of each plasmid is shown. For the HIV provirus, the coding region of viral proteins, including the accessory proteins, is shown. The splice donor site (SD) and the packaging signal (ψ) are indicated. In the packaging construct pCMVΔR9, the reading frames of Env and Vpu are blocked (X). In the Env-coding plasmid, the coding region of 4070a amphotropic MLV envelope is flanked by a MLV LTR and a SV40 poly(A) site. The VSV G coding region is flanked by the CMV promoter and a poly(A) site. In the transfer vector pHR′, the *gag* gene is truncated and out of frame (X), and the internal promoter CMV is used to drive expression of either β-galactosidase (*lacZ*) or luciferase cDNA. The Rev responsive element (RRE) and splice acceptor site (SA) are shown.

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assayed for transduction frequency on growing 208F rat fibroblasts (*21*). HIV-based β-gal vectors yielded titers of 0.8 ($\pm$1.7) $\times$ $10^5$ ($n$ = 3) transducing units (TU) per milliliter with the MLV(Ampho) envelope and 4 ($\pm$1.5) $\times$ $10^5$ ($n$ = 6) TU/ml with the VSV envelope. These titers are comparable with those obtained with MLV-based vectors produced by the same method—$10^5$ TU/ml with its own envelope, and 5 $\times$ $10^5$ Tu/ml when pseudotyped with the VSV envelope—and significantly higher than those previously reported for other HIV-based vectors (*17*, *22*). Potentially contributing to this increased efficiency is the incorporation of accessory HIV-1 genes into the packaging construct, including *nef* that markedly enhances virion infectivity (*23*).

The HIV-derived vector system used here is devoid of helper virus per se. Furthermore, the use of a three-plasmid combination and of a heterologous envelope, as well as the removal of multiple cis-acting sequences from the packaging vector, makes it unlikely that a replication-competent recombinant would be generated. The potential transfer of packaging functions from producer to target cells was assayed by testing for the production of the *tat* and *gag* gene products in vector-transduced cells. Neither protein was detected, which, considering the sensitivity of the assays we used (*24*), implied that the transfer of packaging functions was at least three orders of magnitude less efficient than that of vector sequences. Furthermore, conditioned medium from serially passaged transduced cells did not transfer the reporter gene to naïve cells (*24*).

HIV- and MLV-derived vectors were compared for their ability to transduce cells blocked at various stages of the cell cycle. HeLa cells were growth-arrested at the $G_1$-S boundary or at the $G_2$ phase of the cycle by aphidicolin treatment or gamma irradiation, respectively (*25*). The arrested state of the cells at the time of infection was verified by propidium iodide staining of the DNA and by flow cytometry (*18*). An HIV-based retroviral vector expressing β-gal was as efficient at transducing $G_1$-S– and $G_2$-arrested as proliferating HeLa cells, whereas its MLV counterpart was only 5 to 8% as effective (Table 1). The wider variability observed in the transduction of HeLa cells arrested by gamma irradiation was perhaps due to the cytotoxicity of the treatment.

To test whether the HIV-based vector integrates in the host cell genome, we used packaging constructs carrying mutations that inactivate integrase. HIV-1 mutants in which the expression of integrase is abrogated by the introduction of a stop codon at its 5′ end do not reverse transcribe their genome efficiently (*26*). When this mutation was introduced into the packaging construct, it completely prevented transduction by the resulting vector particles. Furthermore, whereas a β-gal vector made with the wild-type packaging construct had a transduction efficiency of 940 TU per nanogram of p24 in growing or $G_1$-S–arrested cells, a single amino acid change [from aspartic acid to valine at position 64 (D64V)] in the HIV-1 integrase sequence, previously demonstrated to severely decrease the activity of this enzyme but not to affect any other step of infection (*27*), reduced the efficiency to 54 and 130 TU per nanogram of p24 in growing and G1-S–arrested cells, respectively (*28*). Efficient gene transfer in both settings was thus dependent on reverse transcription as well as integration. Taken together, these results indicate that the unique features of HIV can be transferred to a replication-defective retroviral vector, allowing transduction of nonproliferating cells.

To test the transduction of cells arrested in $G_0$, we grew cultures of rat 208F fibroblasts to confluence and then maintained them in $G_0$ by density-dependent inhibition of growth in the presence of dexamethasone (*3*). The HIV-based vector was significantly more efficient than its MLV equivalent. However, its transduction rate decreased as a function of time between growth arrest and infection (Table 1). Cells growth-arrested for 4 days were transduced at levels that were 45% of those observed in dividing cells. However, in cells that had been maintained in $G_0$ for 15 days, the relative transduction decreased to 17%. The MLV-based vector was significantly

**Table 1.** Relative transduction of cells at different stages of the cycle by HIV- and MLV-based vectors. Results are expressed relative to the transduction obtained by the vector in growing cells. Multiplicity of infection was matched for both vectors. Abbreviations: arr., arrested; d, days; repl., replated.

| Infected culture | Tranduction efficiency | |
|---|---|---|
| | HIV-based vector | MLV-based vector |
| HeLa cells* | | |
| Growing | 1 | 1 |
| $G_1$-S-arr. | 0.97 ± 0.02 | 0.05 ± 0.01 |
| $G_2$-arr. | 0.71 ± 0.22 | 0.08 ± 0.01 |
| 208F cells† | | |
| Growing | 1 | 1 |
| $G_0$-arr. 4 d | 0.45 ± 0.02 | 0.11 ± 0.01 |
| $G_0$-arr. 7 d | 0.29 ± 0.02 | 0.03 ± 0.02 |
| $G_0$-arr. 11 d | 0.23 ± 0.01 | 0.02 ± 0.01 |
| $G_0$-arr. 15 d | 0.17 ± 0.01 | 0.01 ± 0.01 |
| 208F cells‡ | | |
| Growing | 1 | 1 |
| $G_0$ | 0.08 ± 0.02 | 0.05 ± 0.02 |
| $G_0$ repl. 2 d | 0.50 ± 0.03 | 0.08 ± 0.02 |
| $G_0$ repl. 4 d | 0.43 ± 0.04 | 0.08 ± 0.02 |
| $G_0$ repl. 8 d | 0.46 ± 0.07 | 0.08 ± 0.02 |

*Human HeLa cells were arrested in $G_1$-S by aphidicolin treatment or in $G_2$ by exposure to 40 grays (1 gray = 100 rads) of gamma radiation (*25*) and infected with β-gal vector pseudotyped with MLV (Ampho) envelope. Transduction was scored by X-Gal staining of the cultures 48 hours after infection. Results are the mean ± SEM determination from four experiments. †Rat 208F fibroblasts were plated at low density and either infected the following day (growing) or grown to confluence, switched to medium containing 5% calf serum and 2 μM dexamethasone (*3*), and further incubated for the indicated number of days (d) before infection with luciferase vectors pseudotyped with VSV G protein. Transduction was scored by measuring luminescence in cell extracts 48 hours after infection. Results are the mean ± SD of replicated determinations from a representative experiment of a total of five performed. ‡Rat 208F fibroblasts either growing or arrested in $G_0$ for 3 weeks were infected with β-gal vectors pseudotyped with the MLV (Ampho) envelope. Transduction was scored by X-Gal staining either 48 hours after infection (growing and $G_0$) or 48 hours after replating (repl.) at low density $G_0$ cultures trypsinized at the indicated days after infection ($G_0$ replated *X* d). Results are expressed relative to the number of blue cell foci obtained by infecting growing cells and are the mean ± SD of replicated determinations from a representative experiment of a total of four performed.

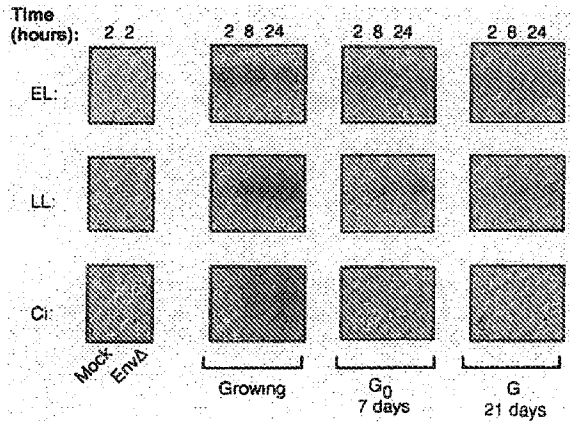


**Fig. 2.** Reverse transcription and nuclear import of the HIV-based vector genome in fibroblasts growing or arrested in $G_0$. Cultures of 208F fibroblasts were plated at low density and either infected the following day (growing) or grown to confluence and further incubated for the indicated number of days ($G_0$ *X* days) before infection with HIV-based luciferase vector pseudotyped or not (ΔEnv) with VSV envelope. At the indicated time in hours after infection, cells were lysed and assayed by PCR with primers specific for various products of reverse transcription, as previously described (*9*, *39*). A sample of the PCR reaction was analyzed by Southern (DNA) blot with a $^{32}$P-labeled HIV proviral DNA probe. EL, early products (strong stop DNA); LL, late linear products (generated after the second template switch); Ci, two-LTR circles (formed in the nucleus).

more affected by the growth arrest. In its case, the residual transducing activity reflected the fraction of cells still undergoing division, as assessed by propidium iodide staining of the cell DNA followed by flow cytometry (*29*). Whereas vector particles entered $G_0$-arrested and dividing cells with comparable efficiencies (*30*), they were significantly defective for reverse transcription in $G_0$ cells (Fig. 2), which resembles a phenomenon observed in HIV-infected quiescent T lymphocytes (*31*). Nevertheless, a stable transduction intermediate must have been established, because replating and proliferation of $G_0$ cells up to 8 days after infection revealed titers as high as 50% of those obtained in dividing cells (Table 1). In contrast, inducing cell division even 1 day after inoculation did not rescue the MLV-derived vector. The generation of a stable infection intermediate by the HIV-based vector offers an advantage for delivering genes into targets such as hematopoietic stem cells. Indeed, it may alleviate the need for inducing the proliferation of these cells ex vivo, a manipulation that can affect their pluripotentiality.

The decreased transduction efficiency of the HIV vector in $G_0$-arrested fibroblasts may partly reflect suboptimal concentrations of intracellular deoxynucleotides (*32*). Whether a similar limitation would preclude gene transfer into terminally differentiated primary cells could not be inferred from these observations and was therefore assessed directly. The HIV-based luciferase vector, pseudotyped with the VSV G protein, was tested for its ability to transduce human monocyte-derived primary macrophages (*33*). Significant levels of luciferase activity were detected in an envelope-dependent manner (Table 2). In contrast, only background levels of luciferase activity were measured in macrophages inoculated with a comparable VSV G–pseudotyped MLV-based vector (*34*). To rule out that the HIV vector was infecting a small proportion of macrophages that were proliferating, we generated mutant packaging constructs where Vpr and the nuclear localization signal (NLS) present in the MA protein were inactivated (*35*). At least one of these two elements is essential for viral infection in macrophages, because they mediate nuclear import of the HIV preintegration complex (*7–9*). A vector assembled from a mutant packaging construct in which both Vpr and the MA NLS are inactivated was severely reduced in its ability to transduce macrophages (Table 2). Similarly, NLS peptide treatment prevented transduction by a vector produced from a Vpr-defective packaging construct, thus corroborating the previously demonstrated inhibition of MA-mediated nuclear import of the HIV preintegration complex by this peptide (*9*). Neither MA-Vpr double mutations nor NLS peptide treatment affected the ability of the vectors to transduce dividing cells (*18*). The requirement for interaction with the cellular nuclear import machinery, together with the lack of significant transduction by the MLV vector, demonstrates that gene transfer by the HIV vector did occur in nonproliferating macrophages and not simply in a small proportion of dividing cells in the culture.

To test if HIV-based vectors can deliver genes in vivo, we injected highly concentrated stocks of HIV- or MLV-based β-gal vectors pseudotyped with VSV G protein bilaterally into the corpus striatum and hippocampus of adult female rat brains (*36*). Seven or 30 days later the brains were removed, sectioned, and processed for immunocytochemistry. Analysis with the light microscope showed no pathological change in the injected areas of the brains, except for a limited deposit of debris and lining-up of scavenger cells along the needle tract in brains examined 1 week after injection. These findings were even less apparent 1 month after the injection. Areas of β-gal–positive cells were detected surrounding all injected sites for both HIV-based and MLV-based vectors. In brains injected with the HIV-

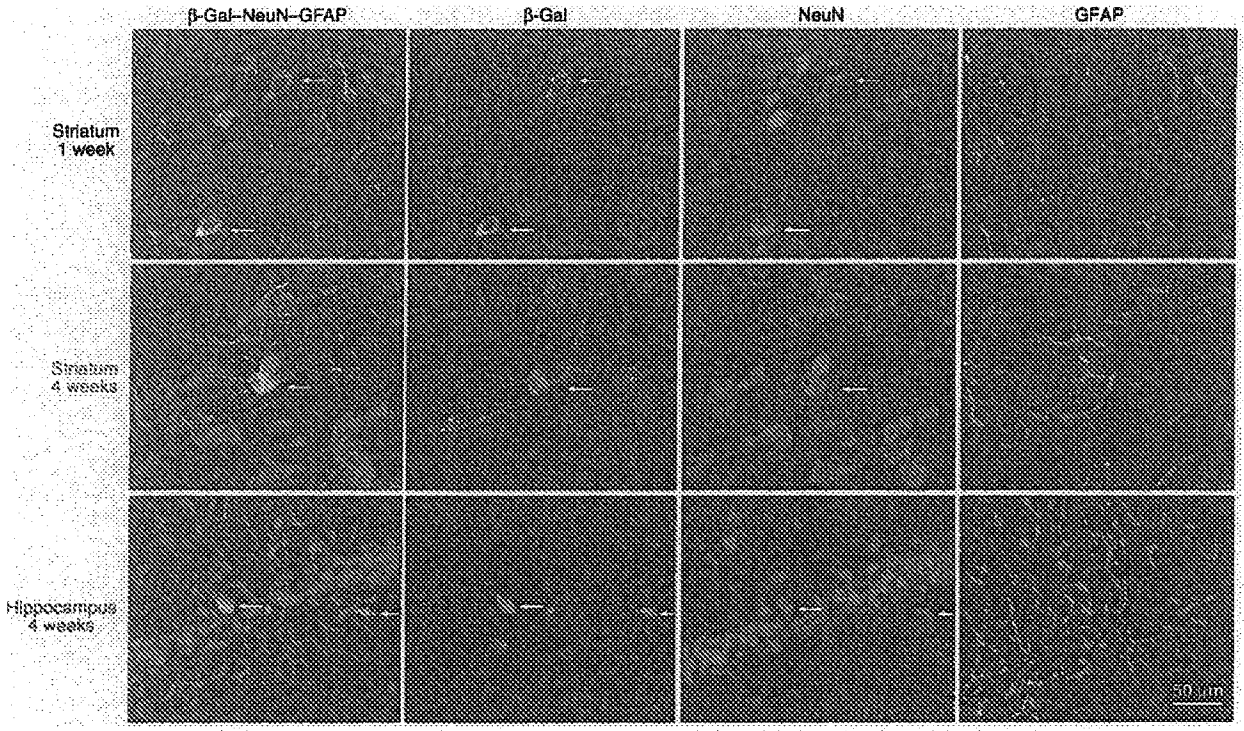


**Fig. 3.** The in vivo transduction of adult rat neurons. Confocal microscope images of sections from brains injected with HIV-based β-gal vectors stained by immunofluorescence for β-gal, NeuN, and glial fibrillary acidic protein (GFAP). The images obtained from each individual staining and from their overlap are shown, as indicated on the top. Representative fields of the area surrounding the injection site are shown for a section from striatum 1 week and 4 weeks and from hippocampus 4 weeks after injection of the vector. Several cells doubly labeled for β-gal and NeuN (arrows) are evident in the sections. The overall pattern was reproduced in all five animals (three examined after 7 days, and two after 30 days) injected with the HIV-based vector.

**Table 2.** Transduction of human monocyte-derived macrophages. Primary cultures of human macrophages prepared from different donors were incubated with HIV-based luciferase vectors pseudotyped with VSV G protein and generated either from wild-type (pCMVΔR9) or mutant packaging plasmids carrying inactivating mutations in the *vpr* gene (ΔVpr) or both in the *vpr* gene and the MA NLS [ΔVpr ΔNLS MA (*33*, *34*)]. Macrophage cultures were incubated with 100 μM of peptide whose sequence corresponded either to the SV40 T antigen NLS to block NLS-dependent nuclear import, or to its reverse, inactive orientation (Rev. NLS), starting 1 hour before and throughout infection, as previously described (*8*). Luminescence was measured in cell extracts 48 hours after infection. Transduction was dependent on active nuclear import of the vector in target cells, as it was inhibited by mutations inactivating Vpr and the MA NLS in the packaging plasmids and when infected cells were incubated with NLS peptide.

| Packaging plasmid | Cell treatment | Luminescence (RLU)* | |
|---|---|---|---|
| | | Donor 1 | Donor 2 |
| Wild type† | — | 0 | 0 |
| Wild type | Rev. NLS | 17,873 | 17,785 |
| Wild type | NLS | 16,225 | 15,322 |
| ΔVpr | Rev. NLS | 8,447 | 9,687 |
| ΔVpr | NLS | 1,141 | 1,348 |
| ΔVpr ΔNLS MA | — | 3,501 | 2,787 |

*Luminescence in relative units above background of 50 μl of infected macrophages extract. †As a control, this plasmid was not pseudotyped with VSV G protein.

based vector, a variety of β-gal–positive cells with a morphology resembling neurons, oligodendrocytes, and astrocytes could be detected (*18*). To further identify the cell types transduced by both vectors, we used confocal microscopy after immunofluorescence staining with antibodies specific for β-gal, glial fibrillary acidic protein (GFAP, a marker for astrocytes), and NeuN (a marker for terminally differentiated neurons) (*37*). Sections from brains injected with the MLV-based vector contained cells either labeled only for β-gal or for both β-gal and GFAP (*18*). The MLV vector was unable to transduce neurons because no cells labeled for both β-gal and NeuN were detected. In contrast, the striatum of animals injected with the HIV-based vector showed multiple cells double-labeled for β-gal and NeuN (Fig. 3, top panel), demonstrating the ability of the HIV-based vector to infect and transduce genes in terminally differentiated neurons. NeuN and β-gal double-positive cells were also detected in the hippocampus of brains injected with the HIV vector. As expected, the HIV-based vector was also able to transduce astroglial cells (*18*). The expression of β-gal in neurons in the striatum and the hippocampus could be detected after a 30-day period, the longest time tested (Fig. 3, bottom two panels).

Our results lend strong credence to the idea that HIV-based vectors transduce genes efficiently and can be used for in vivo gene delivery. Because retroviruses integrate in the genome of the target cells, repeated transduction is unnecessary. Therefore, in contrast to an adenoviral vector capable of in vivo gene delivery, problems linked to the humoral response to injected viral antigens can be avoided (*38*). Furthermore, the vectors described here are replication defective; consequently, the transduced cells lack viral protein that could trigger a cellular immune response. A major goal of our work was to establish a proof of principle that lentiviral vectors can be used for stable in vivo gene delivery in nondividing cells. For human experimentation, it may be more prudent to develop vectors derived from nonhuman lentiviruses such as simian immunodeficiency virus, bovine immunodeficiency virus, or equine infectious anemia virus. We believe that the generation of safe and efficacious lentiviral vectors will significantly advance the prospects of human gene therapy.

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19. A total of 40 μg of plasmid DNA was used for the transfection of a 10-cm-diameter plate of 293T cells in the following proportions: 10 μg of pCMVΔR9, 20 μg of pHR′, and 10 μg of *env* plasmid, as described [C. Chen and H. Okayama, *Mol. Cell. Biol.* **7**, 2745 (1987)]. Conditioned medium was harvested 48 to 60 hours after transfection, subjected to low-speed centrifugation, filtered through 0.45-μm filters, and assayed for p24 Gag antigen by enzyme-linked immunosorbent assay (ELISA) (DuPont). The average vector yield was 50 to 80 ng of p24 per milliliter.
20. MLV-based vectors were produced from transient transfection in 293T cells of the following plasmids. pSLX-CMVLacZ [R. Scharfmann, J. H. Axelrod, I. M. Verma, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4626 (1991)] is a MLV-derived vector carrying a hCMV-driven *E. coli lacZ* gene. The pCL plasmid series carries a hybrid CMV-LTR promoter that allows for CMV-driven transcription in the packaging cell and reconstitution of a functional LTR in the target cell (R. Naviaux, E. Costanzi, M. Haas, I. Verma, in preparation). The luciferase gene was cloned in vector pCLNCX, creating pCLNCLuc. MLV-based vectors with the cognate MLV (Ampho) envelope were produced by the cotransfection of either of the vector plasmids with the amphotropic packaging plasmid pCL-Ampho. VSV G–pseudotyped vectors were produced by the cotransfection of either of the vector plasmids with the MLV *gag-pol* packaging plasmid pCMV-GAGPOL and the VSV G plasmid.
21. Rat 208F cells were infected overnight in six-well plates with serial dilutions of conditioned medium from 293T transient transfectants or with concentrated viral stocks in culture medium supplemented with polybrene (8 μg/ml). The medium was replaced, the cells further incubated for 36 hours, and expression of β-gal scored by X-Gal staining. Titers were calculated by counting the number of foci of blue cells per well and dividing that number by the dilution factor. Transduction of the reporter gene was only observed when the packaging vector and Env-coding plasmid had been cotransfected in 293T cells; no transduction was observed when either plasmid was omitted or when the HIV-based vector was cotransfected with an MLV-

based packaging plasmid or vice versa. Virtually all cells in a well could be transduced when a multiplicity of infection (MOI) >1 was used. When the luciferase vector was used, transduction was assayed by washing the cultures twice with tris-buffered saline (TBS), extracting the cells with 200 μl per well of 0.5% NP-40 in TBS containing 5 mM $MgCl_2$, and assaying a 50-μl sample for luminescence in a luminometer. The HIV-based luciferase vector transduced 930 ± 240 ($n$ = 4) relative luminescence units (RLU) per microliter of infected transfectant-conditioned medium with VSV envelope, and 460 ± 110 ($n$ = 2) RLU with MLV (Ampho) envelope. MLV-based luciferase vector pseudotyped with VSV envelope transduced 920 RLU/μl.

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24. Presence of helper virus and transfer of the HIV *tat* gene were measured by inoculating HeLa P4.2 cells with HIV vector, pseudotyped or not with MLV (Ampho) envelope, and staining with X-Gal after 48 hours. P4.2 cells express CD4 and contain an integrated *lacZ* reporter gene driven by the HIV LTR [P. Charneau *et al.*, *J. Mol. Biol.* **241**, 651 (1994)]. Positive scoring indicated expression of the *tat* gene. The detection limit of the assay was 20 *tat*-transducing units per milliliter of test medium. For comparison, envelope-defective HIV viruses, pseudotyped or not with MLV (Ampho) envelope, were generated by transfecting plasmid pΔER9 in 293T cells. When normalized for p24 Gag antigen, MLV (Ampho)–pseudotyped HIV virus transduced Tat with an efficiency of 630 TU per nanogram of p24 Gag antigen, nonpseudotyped envelope-defective virus had a barely detectable activity of 0.023 TU per nanogram of p24, and no transduction of Tat was detected with a maximal dose tested of HIV vector corresponding to 1.2 μg of p24 Gag antigen, either with or without envelope. On the other hand, when assayed for the transduction of β-gal into naïve HeLa cells, HIV vector pseudotyped with MLV (Ampho) envelope had an average efficiency of 115 TU per nanogram of p24 and of 940 TU/ng when pseudotyped with VSV G. Transfer of the HIV *gag* gene was assayed by measuring p24 Gag antigen in extracts of HeLa cultures serially passaged after infection with the viral equivalent of 1 μg of p24 of both MLV (Ampho)–pseudotyped HIV vector and virus. The detection limit was ≥1 pg per milliliter of extract. Cells infected with envelope-defective, pseudotyped HIV virus consistently gave readings above 20 ng/ml. No Gag antigen was detected in extracts of vector-transduced cells. The same held true when HeLa cells transduced with the pHR'-LacZ vector were selected for β-gal expression by live fluorescence-activated cell sorting after fluorescein-di-β-D-galactopyranoside (FDG) staining [G. P. Nolan, S. Fiering, J.-F. Nicolas, L. A. Herzenberg, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2603 (1988)]. More than 65% of selected cells expressed the marker gene and were serially passaged. Their supernatant scored negative both for p24 Gag content and for transfer of the *lacZ* gene to 208F fibroblasts.
25. For infection of growing cultures, HeLa cells (American Type Culture Collection) were seeded at 1.6 × $10^6$ cells per well in a six-well tray the day before infection. $G_1$-S–arrested cultures were prepared by seeding 2 × $10^5$ cells per well 2 days before infection and adding aphidicolin (15 μg/ml) 24 hours before infection and then daily throughout the experiment. Aphidicolin inhibits DNA polymerases α and δ [J. A. Huberman, *Cell* **23**, 647 (1981); J. J. Byrnes, *Mol. Cell. Biochem.* **62**, 13 (1984)], thereby arresting cells in the $G_1$-S phase of the cycle. $G_2$-arrested cells were exposed for 20 min to a $^{61}$Co source calibrated at 2 grays per minute (1 gray = 100 rads) 1 day before infection and seeded at 4 × $10^5$ cells per well. The gamma radiation induces double-strand DNA breaks, thus blocking cells in $G_2$ [M. B. Kastan *et al.*, *Cell* **71**, 587 (1992)]. Similar results were obtained for all MOIs tested (from 0.001 to 1) and for the transduction of luciferase. Virtually all cells in a well could be transduced with MOI > 1 both when growing and when growth-arrested.
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28. Mutant packaging plasmids were constructed by substituting in pCMVΔR9 a Bcl I–Sal I cassette containing the mutations from plasmids pΔINR8 (*9*) and pHIV-Hygro-D64V (*27*). Although residual transducing activity was scored for the vector assembled from the packaging plasmid carrying the D64V integrase mutation, β-gal–positive cells showed on average significantly weaker staining than those transduced by the wild-type vector and were apparently unable to form foci of stably transduced cells. This is also consistent with the residual activity observed for the D64V integrase mutation in the context of the HIV-1 genome (*27*) and probably reflects expression from episomal DNA.
29. The fraction of cells in S phase was 40 to 50% in growing cells, and from 10 down to 2% after reaching confluence, depending on the elapsed time, as assayed by propidium iodide staining and flow cytometry (*18*).
30. Entry of the HIV-based vector in cultures of 208F cells growing and growth-arrested for 21 days was assayed by measuring the envelope-dependent uptake of p24 Gag protein. Cultures were incubated overnight with 33 ng of p24 Gag antigen of HIV-based vector either pseudotyped or not, washed, trypsinized, and extracted for measuring p24 content by ELISA. Growing cells contained 553 ± 50 pg of p24 after incubation with pseudotyped vector, and 100 ± 25 pg of p24 after incubation with particles with no envelope; $G_0$ cells contained 592 ± 120 pg of p24 after incubation with pseudotyped vector, and 90 ± 15 pg of p24 after incubation with particles with no envelope.
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33. Peripheral blood monocytes were prepared from the buffy coats of healthy donors as previously described (*8*) and cultured in RPMI containing 10% human serum for 2 to 4 weeks before infection. Cultures were infected without or with HIV-based and MLV-based luciferase vectors pseudotyped or not with VSV envelope. For the HIV-based vector, 150 ng of p24 equivalent were used per each inoculum.
34. L. Naldini *et al.*, data not shown.
35. Mutant packaging plasmids were constructed by substituting in pCMVΔR9 either or both of a Bcl I–Sal I cassette from plasmid pΔVprR8, whose *vpr* gene has a truncated reading frame, and a Cla I–Bcl I cassette from $pMA_{KK}27_{TT}R7$, in which two threonines (T) replace two lysines (K) in the NLS of MA. The construction and biological properties of these mutants have been described (*8*, *9*). Occasionally, mutant vectors showed a less pronounced phenotype in the macrophages from one donor, perhaps because of variation in the state of growth arrest of the cells. High-pressure liquid chromatography (HPLC)–purified peptides had the following sequence: PKKKRKVEDPYC (NLS peptide) and PDEVKRKKKPYC (reverse). Abbreviations for the amino acid residues are as follows: C, Cys; D, Asp; E, Glu; K, Lys; P, Pro; R, Arg; V, Val; and Y, Tyr.
36. The details of vector preparation will be published elsewhere (L. Naldini *et al.*, in preparation). After concentration by ultracentrifugation, titers of 1 × $10^6$ to 3 × $10^6$ TU/ml on 208F cells were obtained. All animal procedures were performed according to institution-approved protocols and in a biosafety level 3 environment. Adult female Fischer 344 rats were anesthetized [ketamine (44 mg per kilogram of body weight), acepromazine (0.75 mg/kg), and xylazine (4 mg/kg) in 0.9% NaCl, intraperitoneally], positioned in a stereotactic head frame, and slowly injected with 2 μl of vector stock into the striatum [anteroposterior (AP), +0.2; mediolateral (ML), ±3.5; dorsoventral (DV), −4.5] and hippocampus (AP, −3.5, ML, 3.0; DV, −4.0) bilaterally. Seven or 30 days after injection the rats were deeply anesthetized and perfused with 4% cold paraformaldehyde and 0.2% glutaraldehyde intracardially. The brains were removed, postfixed 24 hours, saturated in 30% sucrose, and sectioned on a freezing microtome (40-μm sections). Light microscopy sections were stained with avidin-biotin peroxidase (Vectastain Elit, Vector Labs) and diaminobenzidine. Immunofluorescence triple labeling was conducted with rabbit antibody to β-gal (anti–β-gal ) (1:1000, Cortex), mouse monoclonal anti-NeuN (1:4), and guinea pig anti-GFAP (1:250, Advanced Immunochemical). Secondary antibodies coupled to fluorescent markers CY5, dichlorotriazinyl amino fluorescein, and Texas Red were used at 1:250 dilution. Slices were mounted with diazobicyclooctane/polyvinyl alcohol mounting medium and analyzed by confocal scanning laser microscopy (Bio-Rad MRTC600). Fluorescent signals were collected, digitally color-enhanced, and superimposed. False-color images were generated electronically with Adobe Photoshop (Adobe System).
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39. For the polymerase chain reaction (PCR) assay, cultures were incubated with vector concentrated by ultracentrifugation and pretreated with deoxyribonuclease I (DNase I) (20 μg/ml for 2 hours at 37°C), washed, trypsinized, and extracted for PCR as previously described (*9*). To adjust for the different content of cellular DNA in growing and confluent cultures, each PCR reaction contained an equal volume (2 μl for EL and LL, 7.5 μl for CI) of both growing and $G_0$-arrested cell extract, either one of which had been infected. The sequence of HIV-specific primers are as follows [positions of nucleotides in the $HIV\text{-}1_{HXB2D}$ sequence, according to L. Ratner *et al.*, *Nature* **313**, 277 (1985), are indicated in parentheses]. LTR5: GGCTAACTAGGGAACCCACTGCTT (496 to 516); LTR6: CTGCTAGAGATTTTCCACACTGAC (635 to 612); 5NC2: CCGAGTCCTGCGTCGAGAGAGC (698 to 677); LTR8: TCCCAGGCTCAGATCTGGTCTAAC (488 to 465 and 9572 to 9549); and LTR9: GCCTCAATAAAGCTTGCCTTGA (522 to 542 and 9606 to 9626). LTR5 plus LTR6 amplifies minus-strand strong stop DNA, LTR5 plus 5NC2 amplifies double-stranded molecules generated after the second template switch, and LTR8 plus LTR9 amplifies two LTR circles. A series of logarithmic dilutions of pHR' plasmid used as a template showed linearity of the PCR reaction over the early time points.
40. We are grateful to G. Nolan, A. Leavitt, and R. J. Mullen for providing reagents; members of the Verma, Gage, and Trono laboratories for helpful suggestions; and J. Stevenson for critical reading of the manuscript. L.N. is on leave from the Institute for Cancer Research, University of Torino Medical School, Torino, Italy, and was supported by the Italian Association for Cancer Research (A.I.R.C.) and currently by the American-Italian Cancer Foundation. U.B. is supported by a fellowship from the Deutsche Forschungs-gemeinschaft, and P.G. from the Swiss National Science Foundation. This work is supported by grants from the NIH and American Cancer Society (I.M.V.); PHS award AI37510 (D.T.); PHS awards AG10435 and AG08514 and Hollfelder Foundation (F.H.G.); and H. N. and Frances Berger Foundation (D.T., I.M.V., and F.H.G.). I.M.V. is an American Cancer Society Professor of Molecular Biology and D.T. is a Pew Scholar. L.N. would like to dedicate this work to the memory of Mauro Naldini.

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EXHIBIT D

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# Mutational Analysis of the DNA Binding, Dimerization, and Transcriptional Activation Domains of MEF2C

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**There are four members of the myocyte enhancer factor 2 (MEF2) family of transcription factors in vertebrates, MEF2A, -B, -C, and -D, which have homology within a MADS box at their amino termini and an adjacent motif known as the MEF2 domain. These factors activate muscle gene expression by binding as homo- and heterodimers to an A/T-rich DNA sequence in the control regions of muscle-specific genes. To understand the mechanisms of muscle gene activation by MEF2 factors, we generated a series of deletion and site-directed mutants of MEF2C. These mutants demonstrated that the MADS and MEF2 domains mediate DNA binding and dimerization, whereas the carboxyl terminus is required for transcriptional activation. Amino acids that are essential for MEF2 site-dependent transcription but which do not affect DNA binding were also identified in the MEF2 domain. This type of positive-control mutant demonstrates that the transcription activation domain of MEF2C, although separate from the MEF2 domain, is dependent on this domain for transcriptional activation through the MEF2 site. MEF2 mutants that are defective for DNA binding act as dominant negative mutants and can inhibit activation of MEF2-dependent genes by wild-type MEF2C.**

The myocyte enhancer factor 2 (MEF2) family of transcription factors comprises a group of transcriptional activators, MEF2A, -B, -C, and -D, that show homology in a MADS (MCM1, Agamous, Deficiens, serum response factor [SRF]) box and an adjacent motif known as the MEF2 domain (6, 25, 31–33, 44, 57; reviewed in reference 49). MEF2 factors form homo- and heterodimers and bind to the consensus site, C/TTA(A/T)$_4$TAG/A, which is found in the control regions of numerous muscle-specific genes and has been demonstrated to be important for skeletal and cardiac muscle gene expression (1, 3, 5, 10–13, 15, 17, 21, 22, 24–27, 36–38, 52, 56, 58). The four vertebrate *mef2* gene products, also referred to as RSRFs (related to serum response factors) (44), have greater than 85% amino acid identity within the MADS domain and an adjacent 27-amino-acid region referred to as the MEF2 domain. This homology in the MADS and MEF2 domains is also present in D-MEF2, the single MEF2 protein in *Drosophila melanogaster* (29, 39, 50), and the characterized MEF2 proteins in *Xenopus laevis* (9, 54).

During embryogenesis, MEF2 transcripts appear initially in precursors of the cardiac and skeletal muscle lineages and are subsequently expressed at high levels in these differentiated muscle cell types (9, 16, 17, 25, 31, 33, 57). Mutations of the *D-mef2* gene in *D. melanogaster* suggest that MEF2 is an essential cofactor for differentiation of skeletal, cardiac, and visceral muscle cells (4, 30). In the absence of D-MEF2, myoblasts are correctly specified and positioned, but they fail to differentiate. These results have led to the notion that MEF2 may be a cofactor for other myogenic regulators that control muscle gene expression in different myogenic lineages (41a).

The exact role of MEF2 in skeletal muscle cells has been unclear. Kaushal and coworkers (23) reported that MEF2 factors have the ability to activate the complete program for skeletal muscle differentiation with an efficiency comparable to that of the myogenic basic helix-loop-helix (bHLH) factors MyoD and myogenin. In contrast, we have found that MEF2 factors lack myogenic activity on their own, but that they potentiate the activity of myogenic bHLH factors (34). This potentiation appears to be mediated by direct protein-protein interactions between MEF2 factors and heterodimers formed between myogenic bHLH factors and E proteins (34). This type of protein-protein interaction allows either type of factor to activate transcription through the other factor's DNA binding site when only one of the factors is bound to DNA.

Despite the importance of MEF2 factors in the control of muscle gene expression, very little is known about the mechanism by which these proteins activate transcription. However, the related MADS-box-containing factor, SRF, has been extensively characterized, and the crystal structure of its DNA binding region has recently been deduced (43). A minimal 91-amino-acid region of SRF containing the MADS box is sufficient for dimerization and site-specific recognition of the serum response element, CC(A/T)$_6$GG (40, 47). The N-terminal region of the MADS box of SRF is predicted to form an α-helix that contacts DNA, while an adjacent hydrophobic region of the MADS box, predicted to form a β-strand, mediates dimerization (43, 47). The MADS box of SRF is also sufficient for transcriptional activation of some SRF-dependent genes because it mediates interactions with accessory factors that activate transcription (20).

A comparison of the MADS boxes of SRF and MEF2 proteins demonstrates a relatively high degree of similarity between amino acids 1 and 38, with more divergence between amino acids 39 and 56 (reviewed in reference 49). It is also interesting that the MADS box in SRF begins at amino acid 141, whereas in all MEF2 proteins, the MADS box is located at the extreme N terminus. Deletion of the N-terminal amino acids preceding the MADS box in SRF results in relaxed DNA binding specificity, such that the MEF2 consensus site can be

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recognized, suggesting that amino acids N terminal to the MADS box influence DNA binding specificity of SRF (48). These data also suggest that MEF2 proteins and SRF are characterized by different structural constraints that result in unique DNA recognition and dimerization functions.

In this study, we characterized the regions of MEF2C that are responsible for transcriptional activation, DNA binding, and subunit dimerization. Our results show that the MADS box is essential for DNA binding and dimerization and that the MEF2 domain plays an important role in DNA binding affinity and an indirect role in dimerization. There are also specific residues within the MEF2 domain that are required for activity of the transcriptional activation region which is located near the C terminus of the protein. Intriguingly, these specific residues in the MEF2 domain do not affect dimerization or DNA binding. The ability of mutants in the MEF2 domain to affect activity of the C-terminal transactivation domain demonstrates that these domains are interdependent. MEF2C mutants that dimerize but fail to bind DNA function as dominant negative mutants and inhibit activation of MEF2-dependent reporter genes in C2C12 myotubes. However, these mutants retain the ability to synergize with myogenic bHLH factors to activate E-box-dependent transcription (34). These results demonstrate that MEF2 factors act through multiple mechanisms to control muscle gene expression.

## MATERIALS AND METHODS

**Assays for DNA binding.** To determine the DNA binding characteristics of either the MEF2C deletion constructs or site-specific mutant constructs, electrophoretic mobility shift assays (EMSAs) were performed. Two microliters of a coupled in vitro transcription-translation product (TNT kit; Promega, Madison, Wis.) was incubated with 40,000 cpm of a $^{32}$P-labeled, double-stranded oligonucleotide corresponding to the consensus MEF2 binding site from the muscle creatine kinase (MCK) gene (17) in the presence of 1 μg of poly(dI-dC) · (dI-dC) for 10 min at room temperature. The EMSA buffer and electrophoresis conditions are described elsewhere (35).

**Site-directed mutagenesis.** Mutations were introduced into the pCDNAI-MEF2C expression vector by rolling-circle PCR as described earlier (18). PCR conditions were as described previously (35). The initial mutant construct was cut with *Hin*dIII and *Xba*I, and the 1.4-kb fragment corresponding to the entire MEF2C cDNA was subcloned into pCDNAI/amp (Invitrogen) and sequenced. Each mutant construct was tested for integrity by in vitro coupled transcription-translation (TNT kit; Promega) in the presence of [$^{35}$S]methionine and then subjected to analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Fig. 3D).

**Transfections and plasmids.** The MEF2C expression vector, described previously (32), contains the 1.4-kb mouse cDNA cloned into the *Hin*dIII-*Xba*I sites of the cytomegalovirus promoter-directed expression vector pCDNAI (Invitrogen). To assess the activities of the MEF2C deletion and site-specific mutants generated with this construct, transient-transfection assays were performed with the MEF2-dependent chloramphenicol acetyltransferase (CAT) reporter construct pE102MEF2×2CAT, which contains two tandem copies of the MEF2 site from the MCK enhancer upstream from the basal promoter of the embryonic myosin heavy-chain gene, which drives expression of the CAT reporter gene (57). This plasmid is responsive to activation by MEF2 proteins. For assessment of dominant negative MEF2 activity conferred by the point mutations R3T and R24L, transfections were controlled by titrating an equal amount of empty vector, pCDNAI, such that the same amount of expression plasmid was used in all cases.

The activity of each of the mutant MEF2C proteins was analyzed by transfection assays performed with 10T1/2 cells grown in Dulbecco's modified Eagle's medium with high glucose and L-glutamine and 10% fetal bovine serum (growth medium). The cells were grown to 60% confluence in 6-cm-diameter plates, transfected by calcium phosphate precipitation for 16 h, washed, and harvested 48 h afterwards. Ten micrograms of the pE102MEF2x2CAT reporter construct (57) was used along with 5 μg of the MEF2C test construct and 1 μg of pRSVβGAL to control for transfection efficiency.

Analysis of the dominant negative MEF2C protein R3T (Arg-3 changed to Thr) in C2C12 cells was performed by transient transfection of confluent plates of fully differentiated myotubes. Cells were grown in growth medium for 2 days until confluent and then switched to differentiation medium, consisting of Dulbecco modified Eagle medium with high glucose and L-glutamine and 2% horse serum, for 6 days. On day 6, the myotubes were transfected with 5 μg of pE102MEF2×2CAT and 3 or 10 μg of a mutant expression vector encoding the R3T MEF2C protein. Forty-eight hours after transfection, cells were harvested and CAT activities were determined in aliquots of extract containing equivalent amounts of protein.

For analysis of the GAL4 fusion proteins, 10 μg of the GAL4-dependent reporter construct pG5E1bCAT (28) was transiently cotransfected into 10T1/2 fibroblasts with 5 μg of the indicated MEF2C-GAL4 fusion construct as described above. The MEF2C-GAL4 fusions were generated by blunt-end cloning of MEF2C PCR-generated DNA segments corresponding to the indicated amino acid sequences into the expression plasmid pSG424 at the *Sma*I site so that the DNA binding domain of GAL4 (amino acids 1 to 147) is fused to MEF2C. Extracts and CAT assays were performed as previously described (35).

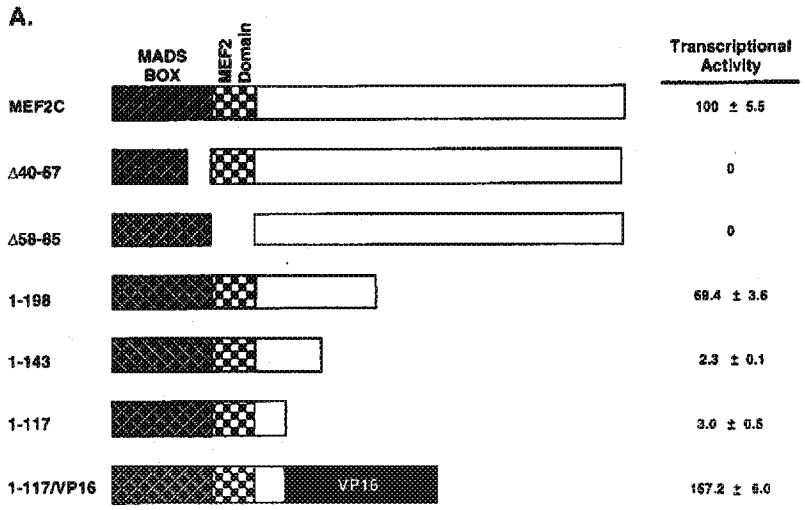
**In vitro translation and immunoprecipitation.** The dimerization potential of each site-directed mutant was assessed by coimmunoprecipitation of in vitro translation products of the indicated full-length mutant construct and a truncated MEF2C construct (amino acids 1 to 105). The truncated MEF2C construct contained a FLAG (Kodak IBI, New Haven, Conn.) epitope at the C terminus so that anti-FLAG antibody could be used for specific immunoprecipitation. In vitro transcription-translation was performed in a total reaction volume of 25 μl with 0.5 μg of each construct. Five microliters of this reaction mix was immunoprecipitated as recommended by the manufacturer (Kodak IBI) in a total volume of 100 μl with 1 μl of anti-FLAG monoclonal antibody and 25 μl of protein G-agarose. The precipitated products were subjected to SDS-PAGE and autoradiography.

**Western blotting (immunoblotting).** The stabilities of the mutant MEF2C proteins were assessed by Western blotting of extracts made from transiently transfected 10T1/2 cells. Constructs encoding wild-type MEF2C and mutants R3T, RKK3-5TNQ, R15L, R17V, KR23,24ID, K25N, K30H, K31L, LSVL35-38QSSM, CDC39-41SDD, LI45,46RN, IF47,48DS, STDMD59-63RAVMH, VLL65-67ASR, and KYTEY68-72ECNDN were separately transfected into 6-cm-diameter dishes of 10T1/2 cells as described above. Only these mutant constructs were assayed because they demonstrated a reduced function compared with wild-type MEF2C. The transfected cells were harvested 48 h later by lysis in 50 μl of 2× loading buffer (125 mM Tris [pH 6.8], 4% SDS, 200 mM β-mercaptoethanol, 20% glycerol, 0.0025% bromophenol blue) and boiled for 5 min. Fifteen microliters of this lysate was electrophoresed on a standard 10% Laemmli gel and blotted to nitrocellulose. Hybridization and washing were performed as described previously (2). Detection of MEF2C was performed by incubation with a primary antibody against MEF2C used at a dilution of 400:1. The secondary antibody was a goat anti-rabbit alkaline phosphatase-conjugated immunoglobulin G-antibody used at a working concentration of 3,000:1 (Boehringer Mannheim, Indianapolis, Ind.). Visualization of alkaline phosphatase activity was performed with 5-bromo 4-chloro-3-indolylphosphate and nitroblue tetrazolium as described previously (2).

Analysis of the stability of the GAL4-MEF2C fusion proteins by Western blotting was performed on extracts of transiently transfected COS cells as described above. The primary antibody was a mouse monoclonal antibody against the DNA binding domain of GAL4 (Santa Cruz Biotechnology, Santa Cruz, Calif.) and was used at a working concentration of 800:1; a goat anti-mouse alkaline phosphatase-conjugated immunoglobulin G secondary antibody (Boehringer Mannheim) was used at a dilution of 2,000:1. The results of both sets of Western blot analyses demonstrated equivalent stabilities of wild-type and mutant MEF2 proteins, suggesting that the differences in transactivation are not due to differences in protein stability (data not shown).

## RESULTS

**The MADS and MEF2 domains lack transcriptional activity.** To identify the regions of MEF2C that were involved in transcriptional activation, we generated a series of deletion mutants and assayed their abilities to support transcriptional activation of a MEF2-dependent reporter gene (pE102MEF2x2CAT) in 10T1/2 cells (Fig. 1A). The reporter gene was efficiently transactivated by MEF2C, whereas the same reporter containing mutated MEF2 sites was not transactivated (not shown). Deletion of amino acids 199 to 465 resulted in a partial loss of transcriptional activity, whereas C-terminal deletions to amino acid 143 or 117 reduced transcriptional activity to a basal level. These results suggested that a strong transcriptional activation domain was located in the C terminus of MEF2C and that the MADS and MEF2 domains, which are located in the amino terminus, lacked transcriptional activation potential. To confirm that residues 1 to 117 retained the ability to bind DNA in vivo, we fused this region of MEF2C to the activation domain of the viral coactivator VP16. This MEF2-VP16 chimera (1-117/VP16) was more potent than full-length MEF2C in activating transcription of the MEF2-dependent reporter, which confirmed that residues 1 to 117 of



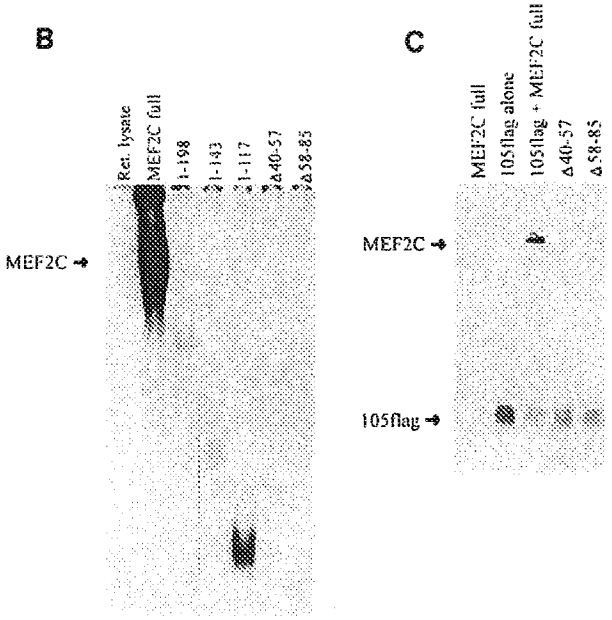




FIG. 1. Transcriptional activity, DNA binding, and dimerization of MEF2C deletion mutants. (A) 10T1/2 cells were transiently transfected with 10 μg of the pE102MEF2x2CAT reporter gene and 5 μg of expression vectors encoding wild-type MEF2C or the indicated MEF2C mutants. Amino acids contained in each mutant are indicated at the left, except for Δ40-57 and Δ58-85, in which the indicated amino acids were deleted. CAT activity in cell extracts was determined as described in Materials and Methods. Values are expressed as the percentage of wild-type activity observed for each mutant and are the averages ± standard errors of at least three experiments. (B) EMSAs were performed with in vitro translation products of wild-type and mutant MEF2C proteins. The MEF2 site from the MCK enhancer was used as a probe. Only the region of the gel containing the shifted complexes is shown. Mutants 1-198 and 1-143 were not expressed as efficiently in vitro, which is why they resulted in relatively faint DNA-protein complexes. We do not know whether their relatively poor expression reflects instability of the proteins, inefficient translation, or insolubility. (C) Coimmunoprecipitation of wild-type and mutant MEF2C proteins was performed with an epitope-tagged MEF2C deletion mutant containing residues 1 to 105. This truncation mutant was cotranslated with wild-type and mutant MEF2C proteins in a rabbit reticulocyte lysate in the presence of [$^{35}$S]methionine. Following immunoprecipitation, labeled proteins were resolved by SDS-PAGE. Neither Δ40-57 nor Δ58-85 showed detectable interaction with MEF2C/1-105FLAG.

MEF2C were sufficient to support DNA binding and dimerization in vivo (Fig. 1A).

The MADS box encompasses residues 1 to 56, and the MEF2 domain encompasses residues 57 to 85. We introduced into MEF2C internal deletions that removed the C-terminal portion of the MADS box (Δ40-57) and the MEF2 domain (Δ58-85). Neither of these mutants was able to activate transcription (Fig. 1A).

The DNA binding activity of each mutant was assessed by EMSA with in vitro-translated protein and the MEF2 binding site from the MCK enhancer as a probe (Fig. 1B). Dimerization potential was also determined by translation of each mutant protein in vitro with an MEF2C truncation mutant containing amino acids 1 to 105 fused to a seven-amino-acid C-terminal FLAG epitope. The resultant heterodimeric complex was immunoprecipitated with FLAG antibody and subjected to SDS-PAGE (Fig. 1C). Wild-type MEF2C was able to dimerize and bind DNA, whereas mutants Δ40-57 and Δ58-85 were not. In light of the role of the MADS box in DNA binding, we anticipated that Δ40-57 would be defective in dimerization and/or DNA binding. These results indicate that the MEF2 domain, which was deleted in Δ58-85, also plays a role in dimerization. Together, these results demonstrate that the dimerization and DNA binding functions in the N terminus of MEF2C can be separated from the transactivating functions in the C-terminal region.

**Mapping of the MEF2C transcriptional activation domain by using GAL4-MEF2C chimeras.** To further define the boundaries of the transcription activation domain of MEF2C, we fused the DNA binding domain of yeast GAL4 (amino acids 1 to 147) to portions of MEF2C and tested the resulting chimeric proteins for their abilities to activate a GAL4-dependent reporter gene in 10T1/2 cells (Fig. 2). Full-length MEF2C fused to GAL4 (GALM2C 1-465) had the strongest transcriptional activity. Deletion of amino acids 175 to 465 (GALM2C 1-174) had only a small effect on transcriptional activity. An intermediate deletion of amino acids 198 to 465 (GALM2C 1-198) resulted in a greater decrease in transcriptional activity, suggesting the presence of an inhibitory domain between amino acids 175 and 198. Further C-terminal deletions to amino acid 143 or 93 (GALM2C 1-143 or GALM2C 1-93) resulted in a complete loss of transcriptional activity. These results are consistent with the deletion analyses of MEF2C and

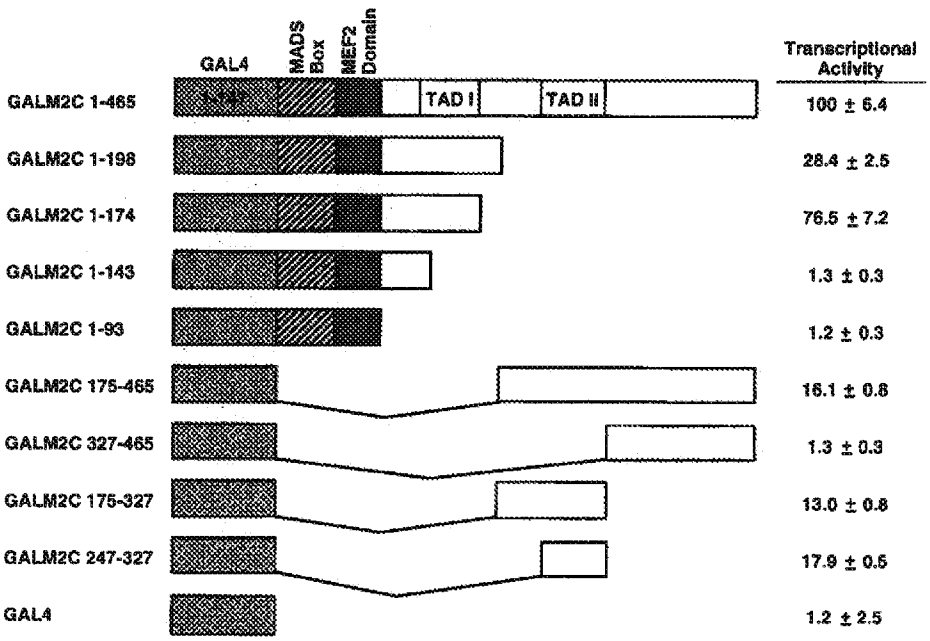



FIG. 2. Transcriptional activity of GAL4-MEF2C chimeras. 10T1/2 cells were transiently transfected with 10 μg of the pG5E1bCAT reporter gene and 5 μg of expression vector encoding the indicated GAL4-MEF2C chimeras. Amino acids of MEF2C contained in each mutant are indicated at the left. CAT activity in cell extracts was determined as described in Materials and Methods. Values are expressed as the percentage of GAL4-MEF2C activity observed for each mutant and are the averages ± standard errors of at least three experiments. TAD, transcription activation domain.

demonstrate that the MADS and MEF2 domains cannot activate transcription alone.

The more C-terminal regions of MEF2C were analyzed further by fusing a series of internal regions of MEF2C to GAL4 (Fig. 2). The region between amino acids 175 and 465 (GALM2C 175-465) could activate transcription. Dissection of this region showed that amino acids 175 to 327 (GALM2C 175-327) retained the ability to activate transcription, while a more C-terminal region (GALM2C 327-465) was inactive. Further deletions showed that transcriptional activation was directed by the region from amino acids 247 to 327 (GALM2C 247-327). Thus, the C-terminal transcription-activating region appeared to be composed of at least two subdomains that could activate transcription independently, one region between residues 143 and 174 and the other between residues 247 and 327.

To control for possible differences in transcriptional activities due to differential stability of one or more of the proteins analyzed, the expression of each protein was examined in extracts of transfected cells by Western blotting with an antibody directed against the GAL4 DNA binding domain. These experiments showed that all of the constructs were expressed at comparable levels (not shown). This finding suggests that the differences in transcriptional activity among the different proteins did not result from differences in expression or stability but reflected regions responsible for activation or repression of transcription.

**Mutational analysis of the MEF2C DNA binding domain.** The deletion mutations demonstrated that amino acids 1 to 117 of MEF2C were sufficient for DNA binding and dimerization, consistent with previous studies of MEF2A (RSRFC4) (44). To map more precisely the specific residues that mediate these activities, we mutated the conserved amino acids within the MADS and MEF2 domains within the full-length MEF2C protein. Mutational analysis of SRF has shown that the first 31 residues of the MADS box mediate DNA binding (41, 47). This region of SRF adopts an α-helical conformation with the basic residues making major and minor groove contacts with the DNA binding site (43). Within the corresponding region of MEF2C, there are 11 basic amino acids which may be involved in DNA-protein interactions. We therefore systematically mutated all of these residues either singly or in combinations to determine which might be required for DNA binding (Fig. 3A). Replacement of Arg-3, Lys-5, Arg-24, Lys-30, and Lys-31 with noncharged amino acids resulted in a complete loss of DNA binding activity (Fig. 3B) without a loss in dimerization potential (Fig. 3C). With the exception of mutant K30H, each of these mutations also resulted in a complete loss in transcriptional activity. The K30H mutant reproducibly transactivated at approximately 20% of the level of the wild-type protein despite an apparent lack of DNA binding capacity in vitro. The explanation for this discrepancy may be in the subtle differences in conditions between the in vitro and in vivo assays, such that K30H may retain modest DNA binding capacity in vivo.

Mutagenesis of residue Lys-4, Arg-15, Arg-17, Lys-23, or Lys-25 resulted in a partial loss of DNA binding capacity without a loss in dimerization potential. These mutations also led to a decrease in the ability to activate the MEF2-dependent reporter gene. Mutation of Arg-10 had no effect on DNA binding capacity and a minimal effect on activation potential. In vitro transcription and translation reactions were performed for each MEF2C construct in the presence of [$^{35}$S]methionine, and the products were subjected to SDS-PAGE (Fig. 3D). The results demonstrate that all proteins are equally stable and that the differences in DNA binding activity result specifically from the designated amino acid substitutions. Together, the results demonstrate that virtually every basic amino acid within the N-terminal MADS-box region from amino acids 1 to 31 of MEF2C is critical for protein function. That all of these basic amino acids are invariant in MEF2 proteins from human,

mouse, chicken, frog, *Drosophila* and *Caenorhabditis elegans* cells also suggests that they are functionally important.

Arg-17, Lys-23, Arg-24, Lys-30, and Lys-31 are highly conserved in all known MADS-box proteins. The amino acids corresponding to Arg-17, Lys-23, and Arg-24 in SRF are essential for DNA binding of SRF (47). Simultaneous mutation of Lys-30 and -31 also diminishes DNA binding by SRF. That mutants R17V and K23T retained residual DNA binding suggests that there are subtly different structural requirements for DNA binding by MEF2 factors and SRF.

Noncharged amino acids were substituted for each of the N-terminal MADS-box mutations so as not to disrupt the α-helical structure that is predicted to form in this region. Each of the amino acid substitutions that resulted in a significant decrease in transactivation was tested for stability in vivo by Western blotting. Western blotting was performed on extracts from 10T1/2 cells transfected with constructs encoding wild-type or mutant MEF2C proteins. The results showed no differences in protein stability for any of the constructs tested (see Materials and Methods), suggesting that the differences in transactivation shown in Fig. 3A are not the result of differences in protein stability (data not shown).

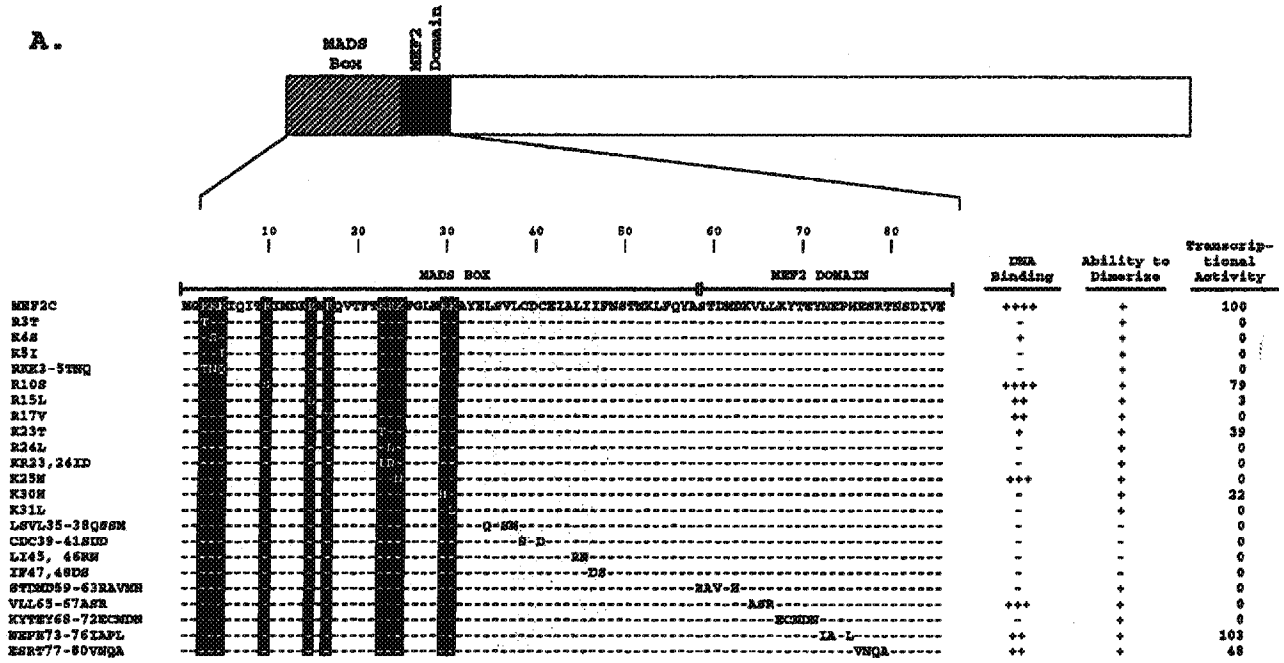
**Identification of the MEF2C dimerization domain.** The amino-terminal α-helical region of the MADS box is followed by a region in SRF and MEF2 factors (amino acids 28 to 56) that is predicted to adopt a β-sheet conformation. The crystal structure of SRF reveals that this region extends away from the DNA and makes relatively few DNA contacts (43). Within this region, there is a stretch of hydrophobic amino acids from residues 35 to 48. All known MADS-box proteins contain relatively similar regions of conserved hydrophobicity. To investigate the role of this region, we mutated the majority of these hydrophobic residues and tested the resulting mutants for their abilities to dimerize, bind DNA, and activate transcription. For many of the mutations, charged substitutions were introduced to disrupt the local hydrophobic pocket that is predicted to form in this region. Mutagenesis of groups LSVL35-38, LI45,46, and IF47,48 eliminated DNA binding and dimerization potential (Fig. 3B and C). The loss in DNA binding capacity is presumably due to a failure of these mutants to dimerize and is not due to a loss in protein stability (Fig. 3D and results of Western analyses [not shown]). These results suggest that the identified hydrophobic amino acids are required for dimerization and subsequent DNA binding. Surprisingly, mutation of the two cysteines at positions 39 and 41 (mutant CDC39-41SDD) also resulted in a loss in dimerization potential. This could be the result of a loss in the overall secondary structure of this region, or it could reflect a specific requirement for these residues in dimerization.

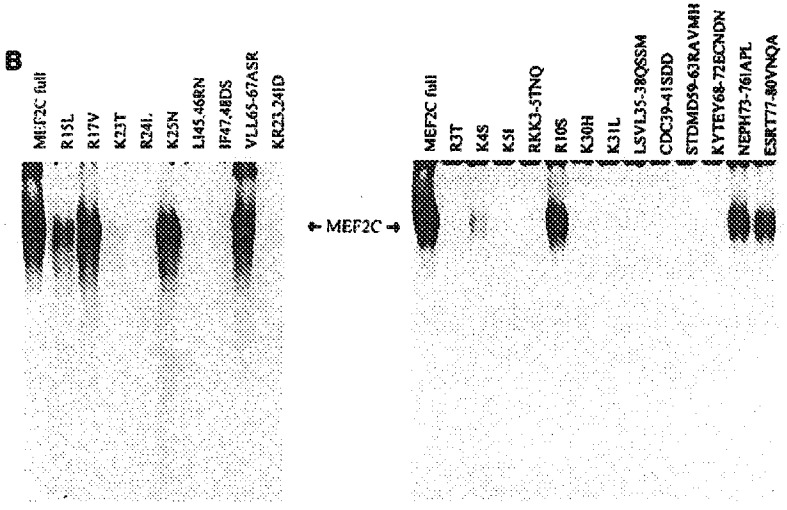
**Role of the MEF2 domain.** The MEF2 domain, which is adjacent to the MADS box, is conserved in and unique to members of the MEF2 family. Deletion mutation Δ58-85, which removed the MEF2 domain, resulted in a complete loss in dimerization and DNA binding capacity (Fig. 1). These results could be interpreted to indicate either that the MEF2 domain is directly required for dimerization or that it simply acted as a permissive region for dimerization directed by the hydrophobic patch in the MADS box. If the MEF2 domain contained specific amino acids that were required for directing dimerization, then mutagenesis of these amino acids should demonstrate this fact. However, if the MEF2 domain was indirectly required as a structural element, then a loss of this domain and subsequent truncation might result in a conformational change that could interfere with dimerization. To distinguish between these possibilities, we introduced a series of block mutations within this region of MEF2C (Fig. 3). None of these mutations had any effect on dimerization, suggesting that the MEF2 domain does not directly specify interactions between MEF2 monomers, but that it is required as a structural element that permits dimerization. Mutations in the MEF2 domain were also performed in blocks of four and five amino acids to scan for a region that might be involved in cofactor interaction and hence have an effect on transcriptional activation. This homologous region in SRF has been shown to interact with cofactors that are required for serum-regulatable expression (see below) (20; reviewed in reference 49).

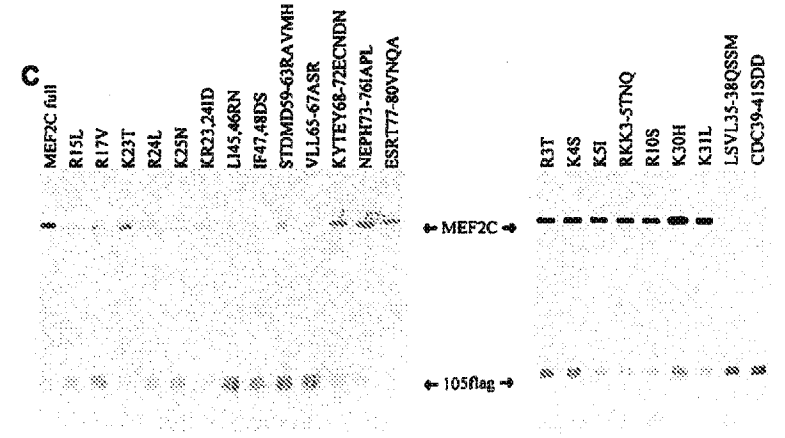
Several mutations in the MEF2 domain impaired DNA binding and transcriptional activity. Mutation of amino acids STDMD at positions 59 to 63 and amino acids KYTEY at positions 68 to 72 eliminated DNA binding activity without affecting dimerization potential (Fig. 3B and C). Both of these mutants also lacked the ability to activate the MEF2-dependent reporter (Fig. 3A). Mutation of amino acids NEPH and ESRT at positions 73 to 76 and 77 to 80, respectively, also diminished DNA binding without affecting dimerization. Taken together, the results of these specific mutations suggest that the MEF2 domain is involved in both DNA binding and dimerization.

Mutation of the hydrophobic amino acids VLL at positions 65 to 67 resulted in only a minor decrease in DNA binding in vitro and had no effect on dimerization potential of the protein. However, this mutant completely lacked the ability to activate transcription. To confirm that this mutant protein was stable and was able to bind DNA in vivo, we performed EMSAs with nuclear extracts from COS and 10T1/2 cells that had each been transiently transfected with the VLL65-67ASR expression vector. DNA binding activity comparable to that of the wild-type protein was observed from extracts of each cell type (data not shown). The inability of this mutant to activate transcription demonstrates that the MEF2 domain is critical for transcriptional activity of the C-terminal transactivation domain in the context of the full-length protein and that DNA binding is necessary but not sufficient for transcriptional activation by MEF2. This result also suggests that the MEF2 domain mediates an additional event required for activation of gene expression.

**Mutation of the MEF2C DNA binding domain generates dominant negative proteins.** Mutations in the MADS box of MEF2C that eliminated DNA binding without affecting dimerization might be predicted to generate dominant negative proteins that can interfere with the activity of wild-type MEF2 proteins by dimerizing with wild-type MEF2 and preventing subsequent DNA binding and transcriptional activation. To test this possibility, we measured the transcriptional activity of wild-type MEF2C in the presence of increasing amounts of the DNA binding mutants R24L and R3T, which fail to bind DNA but retain the ability to dimerize (Fig. 4A). When 10T1/2 cells were transfected with expression vectors encoding these mutants at a 1:1 ratio with either wild-type MEF2C or MEF2A, we observed an ~25% decrease in activation of the MEF2-dependent reporter gene pE102MEF2x2CAT. Higher relative amounts of the mutants resulted in near-complete inhibition of reporter gene expression (Fig. 4A). These results demonstrate that the MEF2C mutants R24L and R3T are capable of acting in a dominant negative manner, presumably because they dimerize in vivo with wild-type MEF2 proteins and form inactive heterodimers. To further characterize the inhibitory activities of these dominant negative mutants, transient-transfection assays were performed with C2C12 myotubes, which contain high levels of endogenous MEF2 DNA binding activity (17). As reported previously, the MEF2-dependent reporter gene was expressed efficiently in C2C12 myotubes (Fig. 4B).


A.
MADS Box
MEF2 Domain
MADS BOX
MEF2 DOMAIN
DNA Binding
Ability to Dimerize
Transcriptional Activity
MEF2C ++++ + 100
R3T - + 0
K4S + + 0
K5I - + 0
RKK3-5TNQ - + 0
R10S ++++ + 79
R15L ++ + 3
R17V ++ + 0
K23T + + 39
R24L - + 0
KR23,24ID - + 0
K25N +++ + 0
K30H - + 22
K31L - + 0
L5VL35-38QSSM - - 0
CDC39-41SDD - - 0
LI45, 46RN - - 0
IF47,48DS - - 0
STDMD59-63RAVMH - + 0
VLL65-67ASR +++ + 0
KYTEY68-72ECNDN - + 0
NEPH73-76IAPL ++ + 103
ESRT77-80VNQA ++ + 48


B
MEF2C full
R15L
R17V
K23T
R24L
K25N
L145,46RN
IF47,48DS
VLL65-67ASR
KR23,24ID
← MEF2C →
MEF2C full
R3T
K4S
K5I
RKK3-5TNQ
R10S
K30H
K31L
LSVL35-38QSSM
CDC39-41SDD
STDMD59-63RAVMH
KYTEY68-72ECNDN
NEPH73-76IAPL
ESRT77-80VNQA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K23T
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IF47,48DS
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VLL65-67ASR
KYTEY68-72ECNDN
NEPH73-76IAPL
ESRT77-80VNQA
← MEF2C →
← 105flag →
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CDC39-41SDD

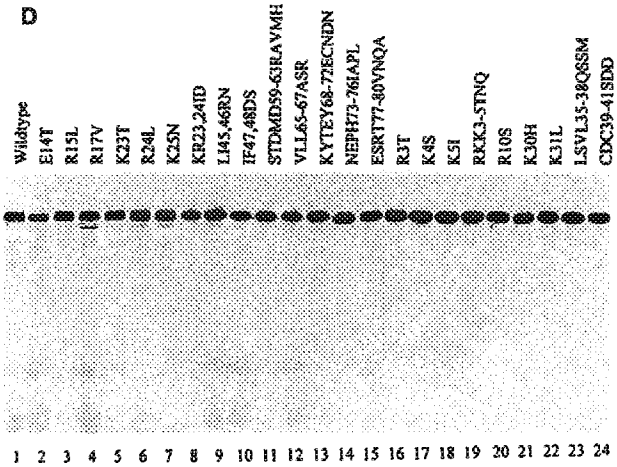


FIG. 3. Transcriptional activity, DNA binding, and dimerization of wild-type and mutant MEF2C proteins. (A) The amino acid sequences of the MADS and MEF2 domains of mouse MEF2C are shown, and the names of mutants are at the left. A dash indicates no change at that position. Basic amino acids within the DNA binding domain are indicated in black, and the hydrophobic dimerization domain is shaded. Relative transcriptional activities determined by using pE102MEF2x2CAT as a reporter in transfected 10T1/2 cells are indicated. Relative DNA binding activities were determined by PhosphorImager analysis and are indicated as ++++ (wild-type DNA binding), +++ (~75% of wild-type binding), ++ (~50% of wild-type binding), + (~25% of wild-type binding), or − (no binding). Dimerization (+) or its absence (−) is also shown. (B) EMSAs were performed with in vitro translation products of wild-type and mutant MEF2C proteins. The MEF2 site from the MCK enhancer labeled with $^{32}P$ was used as a probe. Only the region of the gel containing the shifted complexes is shown. (C) The MEF2C deletion mutant containing residues 1 to 105 fused to the FLAG epitope was cotranslated with wild-type and mutant MEF2C proteins in a rabbit reticulocyte lysate in the presence of [$^{35}S$]methionine. Following immunoprecipitation, labeled proteins were resolved by SDS-PAGE. (D) The various MEF2C mutant constructs were in vitro translated in the presence of [$^{35}S$]methionine and subjected to SDS-PAGE. The results demonstrate similar stabilities for the various products.

However, in the presence of mutants R24L or R3T, expression was reduced by ~90%. No reduction in basal activity was seen for an identical construct containing mutated MEF2 sites (data not shown). This result suggests that the dominant negative proteins R24L and R3T can dimerize with endogenous MEF2 proteins in C2C12 myotubes and inhibit their activities.

To further characterize the effect that was mediated by these dominant negative proteins, EMSAs were performed with extracts of transfected 10T1/2 cells (Fig. 4C). Untransfected 10T1/2 cells showed almost no MEF2-like activity (lane 1); however, transfection of 2 μg of the wild-type MEF2C expression vector resulted in a robust shift (lane 2). If a 10-fold excess of the mutant R3T MEF2C expression vector was cotransfected, an 80% decrease in the shifted band was observed (lane 3). These results demonstrate that the decrease in transcriptional activation directed by these dominant negative MEF2C proteins is due to heterodimerization and subsequent sequestration of wild-type MEF2 proteins in vivo.

## DISCUSSION

Members of the MADS-box family of transcription factors have been identified in plants, the yeast *Saccharomyces cerevisiae*, invertebrates, and vertebrates. The primary amino acid sequences of the MADS boxes from the different members of the family show extensive homology, suggesting a common secondary structure as well as related DNA binding sites (Fig. 5). Indeed, most MADS-box proteins have been reported to recognize the DNA consensus sequence $CC(A/T)_6GG$, which is similar to the consensus sequence for the MEF2 factors $CTA(A/T)_4TAG$ (reviewed in reference 49). There is greater than 85% sequence identity between the MADS and MEF2 domains of MEF2 proteins from human (6, 33, 44, 57), mouse (31, 32), chicken (30a), *Xenopus* (9, 54), *Drosophila* (29, 39, 50), and *C. elegans* (23a) cells. The MEF2 domain is also highly conserved among MEF2 factors, but it is not present in other MADS-box proteins.

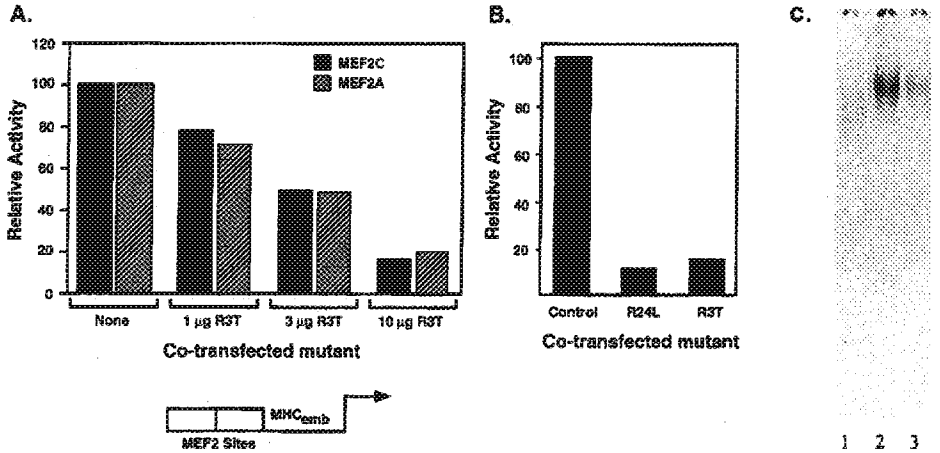


FIG. 4. Inhibition of MEF2-dependent promoters by dominant negative MEF2C proteins. (A) Dose-dependent inhibition of transactivation in the presence of increasing amounts of R3T expression plasmid. 10T1/2 cells were transiently transfected with pE102MEF2x2CAT, 1 μg of MEF2A or MEF2C expression vector, and the indicated amounts of mutant R3T expression vector and empty vector so that the total amount of pCDNAI expression vector remained constant. (B) Inhibition of endogenous MEF2 activity in C2 myotubes in the presence of 3 μg of the indicated mutant expression vectors. Forty-eight hours after transfection, cells in the experiments shown in panels A and B were harvested, and CAT activities were determined in aliquots of extract containing equivalent amounts of protein. In panel B, the degree of reduction in activity was the same when either 3 or 10 μg of dominant negative construct was used, indicating that transient transfection with 3 μg was sufficient to fully saturate endogenous MEF2 activity. No reduction in activity was seen upon transfection with 10 μg of empty expression vector. $MHC_{emb}$, embryonic myosin heavy-chain promoter. (C) EMSA of extracts from transfected 10T1/2 cells with an oligonucleotide corresponding to the MCK MEF2 site. Lane 1, untransfected; lane 2, transfected with 2 μg of wild-type MEF2C expression vector and 20 μg of empty pCDNAI vector; lane 3, transfected with 2 μg of wild-type MEF2C expression vector and 20 μg of MEF2C R3T dominant negative expression vector.

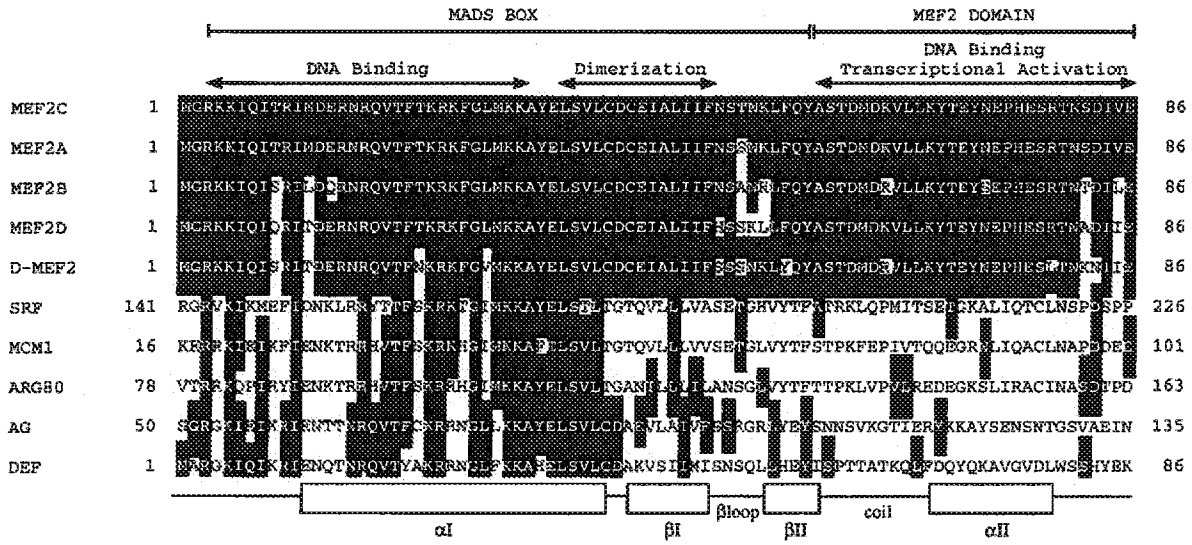


FIG. 5. Identities among MADS-box proteins. Sequences of different MADS-box proteins are shown. Sequences (references) are as follows: MEF2C (32); MEF2A (44); MEF2B (44); MEF2D (31); D-MEF2 (29); SRF (40); MCM1 (42); ARG80 (14); AG (Agamous) (55); DEF (46). Regions of secondary structure in SRF are indicated at the bottom (43). Within the MADS domain, MEF2 factors are similar in secondary structure to SRF, whereas the MEF2 domain does not show predicted structural similarity to the corresponding region of SRF.

**Similarities between the DNA binding and dimerization domains of MEF2 and SRF.** The crystal structure of SRF indicates that the DNA binding region is composed of three structural domains (Fig. 5). The MADS box encompasses a coiled-coil that interacts with DNA and a central β-sheet involved in protein dimerization that also contacts DNA. The amino acid sequence identity between the MADS boxes of SRF and the MEF2 factors suggests that the DNA binding region of the MEF2 factors adopts a secondary structure similar to that of SRF. Immediately C terminal to the MADS box of SRF is a region that is oriented away from the DNA (43) and has been implicated in interactions between SRF and accessory factors (20; reviewed in reference 49). The MADS boxes of SRF and MEF2 proteins have the greatest identity within amino acids 1 to 38, with more divergence between amino acids 39 and 56. The N-terminal region of the MADS box is predicted to adopt an α-helical conformation. The basic residues in the α-helical region of SRF make extensive contacts with the major and minor grooves of the DNA binding site (43). Mutagenesis of this region of MEF2C demonstrated that the majority of the basic amino acids in this region are important for DNA binding.

The C-terminal region of the MADS box (residues 39 to 56) contains a hydrophobic cluster of amino acids predicted to form a β-strand. Our results demonstrate that these residues are involved in dimerization of MEF2 proteins. A similar hydrophobic region has been shown to direct dimerization of SRF (47). However, the spacing and identity of the hydrophobic amino acids are different in SRF and the MEF2 proteins, which may explain the observation that MEF2 proteins do not dimerize with SRF (44).

Mutagenesis of the MEF2 domain demonstrated that it directly influences DNA binding but not dimerization. However, deletion of the MEF2 domain resulted in a mutant MEF2 protein that was deficient in its ability to dimerize, suggesting that this region is structurally required to allow dimerization which is directed by the characterized amino acids in the C-terminal half of the MADS box. Mutations in the MEF2 domain that eliminate DNA binding activity are likely the result of an influence on the MADS box and the manner in which it interacts with DNA. The corresponding region of SRF is oriented distally when bound to DNA and contains a short amphipathic α-helix (αII in Fig. 5), which serves as an interface for dimerization of SRF monomers (43). Yet this region is not in intimate contact with DNA, suggesting that it does not specify DNA interaction. There is no amino acid sequence or secondary structural similarity between these regions of SRF and MEF2, which suggests that the function of the MEF2 domain may not be shared with the corresponding region of SRF. This is consistent with the observation that these regions of SRF and MEF2 factors mediate interactions with different accessory factors (20, 34).

**Interdependence of the MEF2 domain and the transcription activation domains.** Our results demonstrate that the C terminus of MEF2C acts as a transcription activation domain. The first 105 amino acids of MEF2C, which encompass the MADS and MEF2 domains, can dimerize and bind DNA, but this region is unable to activate transcription through the MEF2 site in the absence of a C-terminal transcription activation domain. The C termini of mouse MEF2D and MEF2B and *Xenopus* MEF2A (XMEF2A) are also required for transcriptional activation by these factors (31, 33a, 54). In MEF2C, residues 143 to 174 and 247 to 327 in the C terminus can activate transcription independently. Both of these regions are rich in serine, threonine, and proline residues, which are often found in the activation domains of transcription factors, and correspond to regions of similarity among different MEF2 factors (32). The region from residues 143 to 174 in MEF2C also corresponds to the position of a transactivation domain in XMEF2A, whereas the transcription-activating function contained within residues 247 to 327 of MEF2C appears to be absent from XMEF2A (54).

Mutagenesis of the MEF2 domain yielded an intriguing mutant, VLL65-67ASR, that dimerized and bound DNA but failed to activate transcription. This type of positive-control mutant is similar to mutants that have been generated in the myogenic bHLH factors (7, 13, 45, 53) and indicates that DNA

binding is not by itself sufficient for MEF2 to activate transcription. The ability of mutant VLL65-67ASR to bind DNA without activating transcription suggests that there is an interdependence between the MEF2 domain and the C-terminal transcription activation region. How residues in the MEF2 domain might influence activity of the transcription activation domain, which is located in a separate region of the protein, is unclear. One possibility is that binding of MEF2 to DNA results in an allosteric change in the protein that unmasks the activation domain; such a conformational change might not occur in mutant VLL65-67ASR. Alternatively, the residues VLL at positions 65 to 67 might mediate interactions with other transcription factors, which are disrupted by the mutation. In this regard, other MADS-box proteins such as SRF and yeast MCM1 require interactions with cofactors for activation of their target genes (reviewed in references 19 and 49). The regions of these factors that mediate interactions with accessory proteins are located immediately adjacent to the MADS box, similar to the location of the MEF2 domain.

The MEF2 domain is required for interaction of MEF2 factors with heterodimers formed between myogenic bHLH factors and E proteins (34). We have tested mutant VLL65-67ASR for its ability to interact with myogenin-E12 heterodimers in an in vivo one-hybrid assay and have found that it interacts as efficiently as wild-type MEF2C (data not shown). Similarly, mutant VLL65-67 can synergize with the bHLH region of myogenin to induce transcription through the E-box sequence. Thus, these residues do not appear to affect formation of a MEF2C-myogenin-E12 ternary complex. Whether they might mediate interaction with cofactors involved in general transcriptional initiation remains to be determined.

While residues VLL at positions 65 to 67 of MEF2C are required for transcriptional activation through the MEF2 site, these residues are not required for activity of the C-terminal transcription activation domains when they are fused to the DNA binding domain of GAL4. This observation may indicate that the creation of GAL4-MEF2C fusions constitutively activates the activation domains by altering the conformation of the protein or that this type of fusion protein interacts with different cofactors to activate transcription through the GAL4 binding site. Similar observations have been made with myogenin and MyoD, which require specific amino acids in the basic region to activate transcription through an E-box binding site but not through a GAL4 site when these factors are fused to the GAL4 DNA binding domain (45, 51).

Because MEF2 factors function as homo- and heterodimers and interact with myogenic bHLH factors to regulate muscle gene expression (34), we tested MEF2 mutants for possible dominant negative effects on the activities of wild-type MEF2 factors. Indeed, MEF2 mutants defective for DNA binding interfered with the ability of wild-type MEF2C and MEF2A to activate transcription through the MEF2 site. Dominant negative mutants were capable of dimerizing with wild-type MEF2C to sequester it from binding DNA, as determined by EMSAs of extracts cotransfected with constructs encoding both proteins.

Whereas MEF2 DNA binding mutants cannot activate MEF2 site-dependent transcription alone and can block transcriptional activation by wild-type MEF2 factors, these mutants retain the ability to synergize with the bHLH region of myogenin or MyoD to activate E-box-dependent transcription (34). These results suggest that MEF2 can regulate at least two types of target genes: those which are activated directly by MEF2 and lack E boxes and those that lack MEF2 sites and are activated indirectly by interaction of MEF2 with myogenic bHLH proteins that are bound to E boxes. Although residues 1 to 117 of MEF2C are incapable of activating transcription through the MEF2 site, in the presence of myogenic bHLH factors, this region of MEF2C can efficiently activate transcription (34). These results demonstrate that the transcription activation domain of MEF2C is not essential for MEF2 site-dependent transcription when myogenic bHLH proteins are present. Since MEF2 factors are expressed more widely than myogenic bHLH factors, it will be interesting to determine if there are factors in other cell lineages that can collaborate with the MADS and MEF2 domains of MEF2 to regulate gene expression.

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